Universidade de Aveiro Departamento de Biologia
2017

JOANA FERREIRA DE PAIVA MORAIS SOARES

DISTRIBUIÇÃO DE ANFÍBIOS EM CAVIDADES DO MACIÇO DE SICÓ

AMPHIBIAN'S DISTRIBUTION IN CAVES OF THE SICÓ MASSIF

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AMPHIBIAN'S DISTRIBUTION IN CAVES OF THE SICÓ MASSIF

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Aplicada, realizada sob a orientação científica do Doutor Sérgio Miguel Reis Luís Marques, Investigador de Pós -Doutoramento do CESAM da Universidade de Aveiro e coorientação científica da Doutora Ana Sofia P. S. Reboleira, Professora Associada do Museu de História Natural da Dinamarca, Universidade de Copenhaga e do Doutor Fernando José Mendes Gonçalves, Professor Associado com Agregação do Departamento de Biologia da Universidade de Aveiro.

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agradecimentos

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palavras-chave

maciço calcário Sicó, anfíbios, distribuição, cavidades, dolinas, biomarcadores.

resumo

Algumas espécies de anfíbios (salamandras, sapos e rãs) habitam ambientes cársicos, e são frequentemente encontrados tanto à superfície como em habitats subterrâneos. O presente trabalho aborda ambos através da avaliação da: (a) abundância e distribuição de anfíbios em cavidades do maciço de Sicó e da (b) adequabilidade da água presente em dolinas e exsurgências do maciço de Sicó para as fases iniciais de desenvolvimento de anfíbios.

Apesar de existirem alguns registos de anfíbios em habitats subterrâneos na Europa, em Portugal existe uma grande lacuna no conhecimento acerca destes habitats, em particular nas regiões cársicas. Desta forma, foi feito um levantamento da presença de anfíbios em grutas do maciço de Sicó. Com base nos resultados, foram documentadas duas espécies: *Triturus marmoratus* and *Bufo bufo.* Ambas foram já documentadas em ambientes subterrâneos pela Europa. No entanto, este é o primeiro registo de observação destas espécies em cavidades no centro de Portugal. A presença neste tipo ambiente pode ser justificada pela procura de um local de refúgio com elevada humidade, para se defender de predadores ou ainda por quedas acidentais, uma vez que algumas entradas verticais atuam como armadilhas. Este trabalho demonstra ainda que a presença de anfíbios em cavidades cársicas é subestimada e serão necessários mais levantamentos para contemplar estas questões, tanto no maciço de Sicó como noutras áreas cársicas de Portugal.

Relativamente à superfície e às massas de água das paisagens cársicas, sabe-se que estão ameaçadas pela poluição e contaminação resultantes de actividades como a agricultura e a indústria, entre outras. Devido à escassez de água à superfície, dolinas, lagos, nascentes e exsurgências são essenciais à reprodução de muitas espécies de anfíbios. Tendo em conta estes factores, avaliámos através de ensaios ecotoxicológicos, o efeito de água proveniente de 6 locais distintos do maciço de Sicó, em fases larvares de *Hyla arborea.* Os parâmetros avaliados foram a sobrevivência, o tamanho, a actividade de enzimas antioxidantes (GPx total, GPx Se-dependente, GRed e GST) e também a peroxidação lipídica. De forma complementar foi analisada a presença de 6 pesticidas e foram determinados vários parametros abióticos. Os resultados mostraram que, para dois locais, quer pela deteção de pesticidas, quer pelos resultados obtidos nos biomarcadores, poderão não existir as condições mais adequadas para o desenvolvimento de fases larvares de anfíbios. No entanto, são necessários mais estudos de forma a confirmar estas suspeitas. Considerando ainda a relevância destes locais para a conservação de algumas populações de anfíbios, é necessário realizar mais estudos complementares, nomeadamente avaliando o efeito da sazonalidade nos parâmetros químicos da água e avaliando em fases larvares os efeitos decorrentes de uma exposição *in situ*.

keywords Sicó karst massif, amphibians, distribution, caves, springs, biomarkers.

abstract

Amphibians (salamanders, toads and frogs) are frequently found exploring karst environments, both at the surface and underground. This study aimed to approach both aspects through the evaluation of (a) the abundance and distribution of amphibians in caves of Sicó massif, central Portugal, and (b) the suitability of the water from Sicó massif sinkhole ponds and springs for amphibian's early stages of development.

Despite some studies reporting the presence of amphibians in European underground habitats, in Portugal there is a major lack of knowledge of these ecosystems, namely in the karst region. In order to provide further knowledge, several surveys were carried out in caves of the Sicó massif. Our main results document the observation of two species: *Triturus marmoratus* and *Bufo bufo*. Reports on these organisms are common in the underground habitats in Europe. However, in Portugal this is the first occurrence of both species in caves in central Portugal. Their presence might be due to searching for refuge and high humidity, escaping from predators, and accidental fall into cave entrances that might act as natural pitfalls. This research shows that the presence of amphibians in caves is underestimated and that more caves should be surveyed for the presence of amphibians in this particular massif, but also in other main karst areas in Portugal.

Karst landscapes are threatened with pollution and contamination resultant from activities such as agriculture and industry, among others. Due to the scarcity of water on the surface, sinkhole ponds and springs are essential as breeding sites for these organisms. Considering these factors, we evaluated the effect of water collected from 6 different sites of Sicó massif in early stages of *Hyla arborea*. Through ecotoxicological essays, parameters such as survival, size, activity of antioxidant enzymes (GPx total, Se-dependent GPx, GRed and GST) and also lipidic peroxidation were evaluated. Complementarily, we determined several abiotic parameters and evaluated the presence of 6 pesticides. The results from both biomarkers evaluation and detection of pesticides show that in two sites conditions for larval development of amphibians may not be adequate. However, more studies are necessary to confirm these suspicions. Considering the relevance of these sites for conservation of various amphibian populations, further and complementary studies are necessary, namely for evaluating the effect of seasonality on water's chemical parameters and the resulting effects of *in situ* exposure on larval stages.

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Chapter I

General Introduction

1.1 Karst Massifs of Portugal

Karst landforms refer to a type of geological topography formed by rainwater's dissolution of soluble bedrocks (usually composed of calcium carbonate (CaCO3)) such as limestones, dolomites and marble, having a unique dynamic with the hydrological cycles (Williams 2008). As the water infiltrates underground, carving internal drainage systems, it reemerges at springs (Williams 2008). The karst landscape has a distinctive beauty and ads a "fourth dimension" to typical reliefs. It is characterized by a variety of features such as dry valleys, enclosed depressions, karren fields, lapies, poljes and sinkholes as the exokarst (surface); and also subterranean drainage, resurgences and caves as the endokarst (underground) (Kiesecker 2002; Gunn 2004; Williams 2008). They are important, complex and highly productive environmental systems that provide freshwater aquifers, mineral resources, harbor unique biodiversity and are significant and valuable cultural sites for the study of paleontology, geology, biology, archaeology among many others (Watson et al. 1997).

Despite being relatively small compared to other European countries, the portuguese territory has a very diverse landscape from North to South. A major part of the territory is established on the european hercynian segment, the Iberian Massif – which consists of Paleozoic geological formations of mainly metamorphic and igneous rocks (Dallmeyer & Martínez García 2012), and is divided into three subunits: *Zona Centro-Ibérica* (ZCI), *Zona de Ossa-Morena* (ZOM) and *Zona Sul-Portuguesa* (ZSP). Along with the Mesocenozoic borders (*Orla Ocidental* and *Orla Meridional*) and the Tejo-Sado Cenozoic basin, both formed by sedimentary rocks, they form the four principal geological units in which the territory is divided (Fig. 1) (SNIRH 2017).

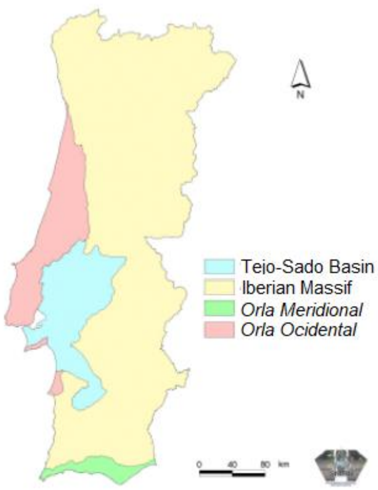

Figure 1. Geomorphologic units of continental Portugal (adapted from SNIRH, 2017).

There are 12 main karst areas in continental Portugal (Fig. 2) (Reboleira 2012). The most typical and extensive ones are located in the Mesocenozoic borders, the *Orla Ocidental* corresponding to Jurassic (Dogger/Malm) limestones and cretaceous sandstones and limestones (Sicó-Alvaiázere, Estremenho, Arrábida, and Montejunto) and the *Orla Meridional* corresponding to Jurassic limestones and dolomites and detrital and carbonate Cretaceous formations (Algarve) (Almeida et al, 1995; SNIRH, 2017).

Karst areas occupy about 5% of national portuguese territory (Cunha & Dimuccio 2014) and they are particularly vulnerable systems whose integrity is dependent upon a balance between land, water, vegetation and soil, and thus need protection and conservation. Several and often diffuse sources of contamination affect karstic landscapes (Gunn 2004). Many economic activities such as agriculture, water management, wastewater effluents, intensive speleological activities and mining activities pose real threats to the integrity and equilibrium of these landscapes (Watson et al. 1997; Lee et al. 2012; Cunha & Vieira 2004; Gunn 2004). Indeed groundwater system's contamination with toxic compounds such as pesticides, heavy metals, nitrates and other pollutants is mainly caused by agriculture, livestock activities and urban sewage effluents (ARH Tejo 2011; SNIRH 2017). Based on EPPNA method (qualitative model for assessing aquifer vulnerability based on lithological groups), the two karstic systems more vulnerable to water contamination are Estremenho and Sicó-Alvaiázere massifs (Pires 2014).

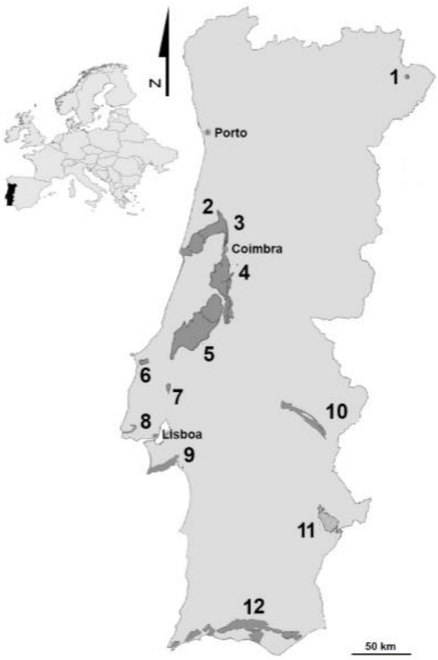

Figure 2. Main karst areas: 1 – Dine e Vimioso; 2 – Outil-Cantanhede; 3 – Mealhada; 4 – Sicó; 5 – Estremenho; 6 – Cesaredas; 7 – Montejunto; 8 – Península de Lisboa; 9 – Arrábida; 10 – Estremoz-Cano; 11 – Adiça-Ficalho; 12 – Algarve (adapted from Reboleira 2012 and Reboleira et al. 2015).

Urbanization and economic revitalization of these mainly rural areas since the 90s has promoted many sports related, leisure and tourism activities, intensifying the environmental frailty (Cunha & Vieira 2004). Because of their economic value, these areas have been targeted with some concern as to preserve historic and cultural patrimony, but not so much concerning their biodiversity and natural resources (Cunha & Vieira 2004).

1.2. Amphibians

1.2.1. Amphibian traits and conservation status

Amphibians have the singularity of having both an aquatic and a terrestrial phase in their lifecycles. In general, reproduction is oviparous and larval stages are aquatic, undergoing metamorphosis to become terrestrial or semi-terrestrial adults (Almeida et al. 2003). Due to their water requirements, water availability or at least high levels of humidity are ecological restrictions to their survival, particularly during reproduction periods (Almeida et al. 2003). In Portugal, amphibians can be found in a wide variety of habitats including mountain, agricultural and forest zones, depending on the specie's specific ecological requirements (Loureiro et al. 2008).

Over the last decades, global herpetological assessments have revealed that over 40% of amphibian populations are decreasing (Stuart et al. 2004) making the establishment of management, protection and conservation plans a top priority. Initiatives such as Rede Natura 2000 in Europe (Mücher et al. 2009; Ostermann 1998) and the compilation of "Livro Vermelho de Vertebrados de Portugal" (Cabral et al. 2005) have induced a boost in collected data for many portuguese species concerning their distribution (Jenkins et al. 2003; Soares et al. 2005; Sequeira et al. 2001; Brito 2003), ecology (Lima et al. 2000), genetics (Elisa 2001; Almeida et al. 2002; Paulo et al. 2002; Marques 2011), behavior (Márquez et al. 2001) and conservation (Malkmus 2005; Santos et al. 2006; Beja & Alcazar 2003; Cruz et al. 2008), among others.

Currently, portuguese amphibian fauna is represented by a total of nineteen species (twelve of them representative of the Anura order and seven of Caudata) of which seven are Iberian endemisms. The Portuguese red list (Cabral et al. 2005) includes *Chioglossa lusitanica* and *Triturus helveticus* species with a vulnerable status and *Discoglossus galganoi* as near threatened.

The main threats to amphibians are the degradation and loss of suitable habitats, particularly due to agriculture and aquaculture and resultant habitat contamination from the use of pesticides, fertilizers, metals and other xenobiotics (Beja & Alcazar 2003; Cunha &

Rego 2005; Ribeiro et al. 2009; Matos et al. 2012; Carpio et al. 2016; Mann et al. 2009; Sánchez et al. 2013); modifications in water flow patterns (Sánchez et al. 2013); pollution and climate change (Sánchez et al. 2013; Cruz et al. 2016); and urbanization (Sánchez et al. 2013). Other threats include introduction of invasive species (Cruz et al. 2008); diseases and pathogen agents such as: viruses (e.g *Ranavirus*) (Soares et al. 2003), bacteria (*Cedecea lapagei* and *Aeromonas hydrophila*) (Soares et al. 2003) and fungi (*Batrachochytrium dendrobatidis*) (Bosch et al. 2001; Rosa et al. 2013). The anti-conservation attitude and deliberate persecution of amphibians due to folklore beliefs, superstitions and even fear, also poses a real threat to certain species, particularly in rural areas (Ceríaco 2012; Sánchez et al. 2013).

1.2.2. Amphibians in karstic landscapes

In Portugal, only a few karst areas are protected through specific legislation: Estremenho massif is partially within the Natural park of Serras de Aire e Candeeiros (PNSAC); Arrábida massif within the Natural park of Arrábida (Dec.-Lei nº 622/76, de 28 de Julho) and Montejunto massif is classified as a protected landscape (Dec. Reg. nº II/99, de 22 de Julho). Others, such as Sicó Massif are integrated in Rede Natura 2000 and considered as a Habitat Directive Site, although no management plan is in action (ICNF, 2017).

Threatened species occurring in Portugal are found mainly in ecosystems such as these rocky areas, mountains, forests and wetlands (Sánchez et al. 2013). Karst areas are habitat to a variety of amphibians and other fauna. Due to surface limited water availability in karstic landscapes, formations such as sinkholes, which by Field (2002) definition are a "basin - or funnel - shaped hollow in limestone, ranging in diameter from a few meters up to a kilometer and in depth from a few to several hundred meters", when filled, permanently or not, with water – becoming sinkhole ponds or doline lakes – are extremely important freshwater resources for local fauna. Amphibians, in particular, are dependent upon ponds for reproduction, some of the species that breed in temporary ponds have the impendent risk of losing entire cohorts if the pond dries before they reach metamorphosis (Vitt & Caldwell 2014).

Because water is more abundant underground compared to the dry surface of karst landscape, sinkhole ponds and doline lakes are not the only karst features that are used by amphibians. They are also commonly found in underground environments (Gunn, 2004; Fellers, 2010; Lunghi et al, 2014). Caves have a generally stable environment withlow fluctuations of temperature, humidity and water availability, making them suitable as thermal refuges (Resetarits 1986), for escaping predators (Manenti et al. 2010; Manenti et al. 2016), for feeding (Sharifi et al. 2014; Fenolio et al. 2006) or even as breeding sites in subterranean pools (Lunghi et al. 2015; Resetarits Jr. & Aldridge 1987; Manenti et al. 2011). Amphibians are ectotherm organisms, which means that the regulation of their body temperature is dependant on external sources (Vitt & Caldwell 2014). Hence, during adverse climacteric conditions some species undergo into hibernation and some hibernation strategies may involve taking refuge under rocks or underground, in which case caves might also be of use.

Amphibians contribute to ecosystem services having important and diverse roles in nutrient cycling, pest control, soil bioturbation and ecosystem engineering, to name a few (Hocking & Babbitt 2014). Due to their generally dualistic nature (aquatic and terrestrial stages), they are particularly fragile to threats such as land use changes and airborne, soil and aquatic contamination. The loss of amphibians can alter freshwater ecosystems and services by, for example, inducing disturbances of the energetic transfer equilibrium between both terrestrial and aquatic systems, also causing disruptions in faunal food chain (Whiles et al. 2006; Collins & Crump 2009). They are often referred and used as bioindicators because of their high vulnerability to changes (e.g. temperature, humidity, pollution/contamination, habitat fragmentation etc.) and low dispersal abilities (e.g. Dunson et al, 1992, Venturino et al, 2003).

Amphibian biogeography

Investigation of spatial distribution patterns - biogeography (Pinho & Ferrand De Almeida 2008) - using modern GIS (Geographic Information System) to collect, manage and map chorological data, was and still is a very important tool in species conservation and sustainable management of biodiversity. Its application allows for example the documentation of species distribution (e.g. Sequeira et al. 2003), changes in populations (range and size) and potential threats (Ribeiro et al. 2009; Sendra & García 2000; Carpio et al. 2016); the analyses of species or communities and environment associations and distribution patterns (Matos et al. 2012; Cunha & Rego 2005; Santos et al. 2006); the production of atlases for scientific or educational purposes (Denoël 2012; Sillero et al. 2005; Soares et al. 2005) and can even be integrated in citizen-science online tools for large scale monitoring (e.g. Na2re Project in Europe) (Sillero, Oliveira, et al. 2014; Sillero, Campos, et al. 2014). Data on geographical distribution is also crucial evidence to enforce the protection of important diversity hotspots and habitats, especially of endangered species, for example in assessing IUCN threatened species categories (IUCN 2012).

Systematic and ecological studies on Portuguese herpetofauna only began in the

70s with Crespo (Crespo 1971, 1972, 1973) and Malkmus's works (Malkmus 1981) (Crespo 2008) and since the 80s, research is integrated in national herpetological associations. The first Portuguese atlas concerning the distribution of herpetofauna, using handmade maps, is published by the national institute for nature conservation (ICN) in 1989 (Crespo & Oliveira, 1989), whilst the first red list of vertebrates from Portugal was published in 1990 (Cabral et al. 2005) and the first field guide "Anfíbios e Répteis de Portugal" with dichotomous identification keys in 2001 (Almeida et al. 2003). By the time of the publication of the third atlas of continental portuguese herpetofauna (Godinho et al. 1999), Geographical Information Systems (GIS) were already being used to produce cartography. By 2004, Loureiro et al. published the first project in the Iberian Peninsula to use GIS from the creation of the database (data collected with GPS) to the cartography (Sillero et al. 2005).

The direct influence of humankind in distribution patterns of biodiversity is well-documented and there are numerous cases worldwide of local and regional population declines/constriction and even extinctions. Periodical local assessments of their occurrence can bring to light relevant information on the impact of altered habitats.

1.2.3. Amphibian ecotoxicology

Among the various causes responsible for the global amphibian decline (see section 1.2.1), exposure to environmental contaminants is one of the most worrisome and is generally regarded as a primary factor for this widespread phenomenon (Stuart et al. 2004; Sparling et al. 2010). Due to biological and ecological traits, such as their high dependence on water from larvae to adults, biphasic life cycle (terrestrial and aquatic) and high permeability of skin and egg capsules, amphibians are particularly susceptible to chemical stressors. Because of these requirements, amphibians generally spend a large proportion of their life cycle in and/or near streams, ponds and temporary pools, normally associated with agricultural sites prone to high contamination with pesticides and fertilizers (Mann et al. 2009). Through runoff, drift or direct overspray, exposure can occur in both water and sediment (eggs and tadpoles) and in land (terrestrial dwelling adults), explaining the high rate of aquatic breeding specie's declines (Todd et al. 2011). Other sources of widespread contamination from a broad range of pollutants include industrial and urban sewage and mining activities among numerous others.

Although still underrepresented in literature, ecotoxicologists have recently and increasingly focused on these organisms to investigate patterns of sensitivity and effects of environmental pollutants (Sparling et al. 2010). Herbicides such as glyphosate, insecticides and fungicides have been proved to be toxic and even lethal to amphibians with well documented effects (Quarles 2015). However, effects in the environment are difficult and complex to fully assess, as many combined factors may potentially make environmental concentrations more or less toxic, for example mixture of pesticides (Sparling et al. 2010). Approaches in the study of ecotoxicology of amphibians usually consist in laboratory acute and chronic assays, with exposures ranging from short duration (96h) up until various weeks. The emphasis over the past decades has been on understanding dose-response associations to determine lethal concentrations and sub-lethal effects (e.g. malformations, behavioral changes, decreased growth and development, etc.) (e.g. Lajmanovich et al. 2003; Fort et al. 2004; Feng et al. 2004). A few standardized protocols have been developed specifically for these organisms, including FETAX (ASTM, 1998) and AMPHITOX (Herkovits and Pérez-Coll, 1999), which are valuable tools for evaluating embryo-larval toxicity of environmental pollutants in amphibians (Mann 2005). More recently, the application of molecular biomarkers allowed a more accurate assessment of sub lethal endpoints of intoxication, long-term effects, bioavailability and interactions between multiple toxic chemicals, among others (see Adams et al. 2001; Venturino et al. 2003; Pechen de D'Angelo et al. 2005).

1.3. Goals and thesis structure

This dissertation is organized into four chapters as follows:

Chapter I

General introduction that frames the objectives and structure of the dissertation. Both studies in chapter II and III were conducted in Sicó karst massif in Portugal with the objective of collecting data on herpetofauna distribution, ecology and ecotoxicology, respectively, for conservation purposes and also highlight the ecological importance of this area.

Chapter II

This study aimed to fill basic information gaps concerning the presence of herpetofauna in cave habitats. To our knowledge, systematic register and mapping of amphibians in caves has never been done in this area. The acquired data contribute with biogeographical and ecological information on amphibian species use of subterranean habitats. The results can be integrated in further studies and may be useful for local species conservation and protection of subterranean sites.

Chapter III

This study was designed to identify the presence of pesticides and metals in Sicó Massif sinkhole ponds and their potential toxic effects on a local aquatic-breeding amphibian species. The study evaluates the effects of exposure to water samples from these potential breeding ponds on lethal and sub-lethal effects (oxidative stress biomarkers) of *Hyla arborea* tadpoles.

Chapter IV

This final chapter aims at providing an overall discussion of the previous chapters (II and III), integrating the results of both studies carried out in Sicó karst massif.

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Chapter II

2. Distribution of amphibians in caves of Sicó massif, Portugal

keywords distribution, amphibians, caves, subterranean habitats, Sicó massif

abstract

Amphibians (salamanders, toads and frogs) are frequently found exploring subterranean environments. Eventhough in Europe there are already some reports of amphibians in underground habitats, in Portugal there is a major lack of knowledge of these ecosystems, namely in the karst region. In order to provide further knowledge over this subject several surveys were carried out in caves of the Sicó massif, Portugal. Based on the surveys, two species were observed: *Triturus marmoratus* and *Bufo bufo*. Reports on these organisms are not new to underground habitats in Europe. However, in Portugal this is the first register of both species in caves in central Portugal. Their presence might be due to searching for refuge and high humidity, escaping from predators, and accidental fall into cave entrances that might act as natural pitfalls, and further surveys are needed to address these issues.

2.1. Introduction

Caves have been related to humankind for millennia, particularly since they were used as shelters for prehistoric humans. Citing Monroe (1970), a cave is “A natural underground room or series of rooms and passages large enough to be entered by a man; generally formed by solution of limestone or a similar artificial opening”, but can also be described more generically as a natural cavity where at least some part of it has total absence of light. Natural karst caves are unique and important expressions of geological patrimony (Williams 2008), they are formed in limestone terrains due to external geodynamic processes that determine the formation of a complex infiltration network and subterranean systems of water circulation and aquifers. Caves are also important habitats that can temporary or permanently harbor a wide variety of organisms such as invertebrates, mammals, birds, amphibians and reptiles (Gunn 2004).

Amphibians, like other vertebrates, have different degrees of specialization and adaptation to hypogean habitats (Sket 2008). For instance, there are a few obligate cavernicolous (troglobiotic) salamanders, that are completely adapted to cave habitats, such as european *Proteus anguinus*, or subtroglophile salamanders (genus *Hydromantes)*, who are dependent on caves only on warmer months (Arntzen et al. 2009; Lunghi et al. 2014), although none of such species occur in Portugal. Epigean amphibian species (toads, frogs and salamanders) are also frequently found in caves (Gunn 2004), in such cases they are considered trogloxenes - organisms that can occasionally be found in subterranean habitats (Sket 2008). Specific features of subterranean habitats such as water availability (contrasting with the limited water on the surface), constant temperatures and humidity are advantageous to certain aspects of their life cycles (Manenti et al. 2009; Manenti et al. 2011; Lunghi et al. 2014). They may actively search caves as thermal refuges (Resetarits 1986), for escaping predators (Manenti et al. 2010; Manenti et al. 2016), for feeding (Sharifi et al. 2014) or for stable freshwater breeding sites (Lunghi et al. 2015; Resetarits Jr. & Aldridge 1987; Manenti et al. 2011; Manenti et al, 2016). Their presence in caves can also be accidental since vertical caves act as natural traps (Sket, 2008). However, some authors suggest that active exploitation of cave habitats (occasional guests) by epigeous species are very often mistaken for accidental occurrence (Lunghi et al. 2014).

There have been several records of non-obligate cave dweller amphibian species found in both natural and artificial caves: *Pleurodeles waltl, Salamandra salamandra*, *Lissotriton boscai* and *Triturus marmoratus* in Spain (Giménez-Lopéz & Guarner Deu 1982; Herrero & Hinckley 2014); *S. salamandra, Triturus vulgaris*, *Triturus cristatus, Bufo bufo, B.*

viridis, Hyla arborea, Rana temporaria, Rana dalmatina in Slovakia (Uhrin & Lesinsky, 1997); *Salamandra salamandra* in Romania (Ianc et al. 2012; Balogová & Uhrin 2014) and Italy (Manenti et al. 2009; Manenti et al. 2010), *Bufo bufo* in Italy (Bonini et al 1999), among others (e.g. Rosa & Penado, 2013; Herrero & Hinckley, 2014).

In continental Portugal, Gilbert and Malkmus (1989) reported evidence of *Chioglossa lusitanica* reproduction inside a mine tunnel and, more than two decades later, Rosa and Penado (2013) reported anuran (*Rana Iberica*) reproduction in a subterranean site in Serra da Estrela. Also, *Chioglossa lusitanica* is known to use subterranean habitats as refuges (ICNF 2017b). Despite the lack of systematic data concerning the presence or distribution of herpetofauna in caves, their occurrence in subterranean sites is a relatively known fact, particularly among speleologists.

To our knowledge, no survey has been done in Sicó massif. Therefore, the main objective of this study was to assess the occurrence of amphibians in caves of the Sicó massif, in order to contribute for the protection of both species and subterranean sites.

2.2. Material and methods

2.2.1. Study site

Sicó massif (figure 3) is considered one of the main karst areas in the *Orla Mesocenozóica Ocidental*, in central Portugal, occupying an area of about 430 km^2 (Vieira & Cunha 2006). It is classified as Site of Community Importance (SCI) integrated in Natura 2000, since 2008 (ICNF, 2017a) (Fig. 3 and 4). Under the influence of a Mediterranean climate under Atlantic influence, this massif covers a set of relatively low hills, mountains (Sicó: 553 m and Alvaiázere: 618 m), uplands and other diverse limestone associated habitats covering Alvaiázere, Ansião, Condeixa-a-Nova, Penela, Pombal and Soure regions (Vieira & Cunha 2006). These sedimentary units are arranged in a monoclinal structure towards west and intersected by significant faults (NNE-SSW to N-S and ENE-WSW) (Cunha & Dimuccio, 2014). It includes four rock formations of great importance: *Coimbra* group; a set of dolomites, dolomitic and inferior Jurassic limestones; medium Jurassic dolomites formations (Póvoa de Lomba, Degracias formation and Senhora da Estrela formation) and the Quaternary formation of Condeixa (tufts of limestone and travertines) (see Cunha et al, 2010 and references therein).

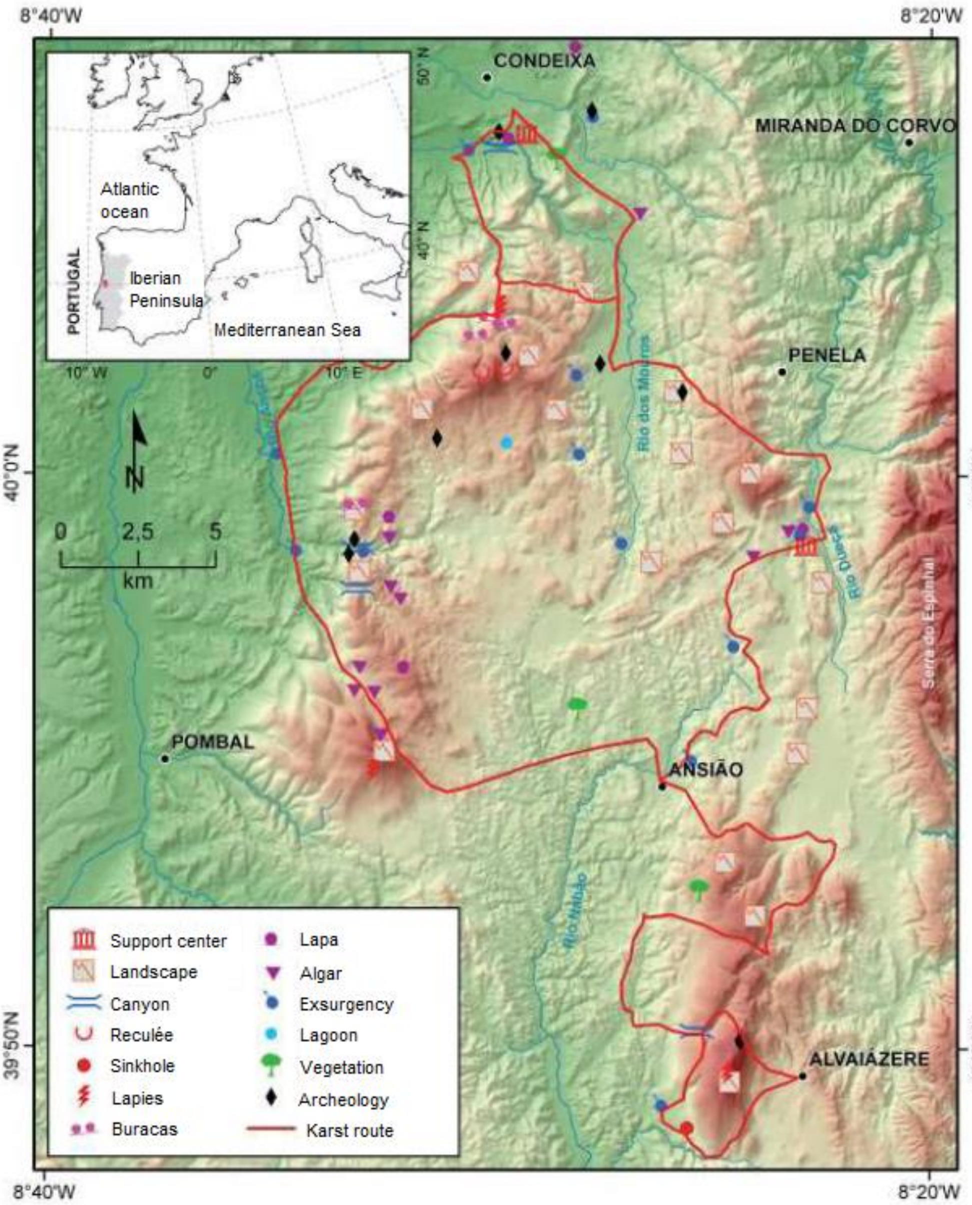

Figure 3. Sicó karst and main patrimony elements (source Cunha & Dimuccio, 2014). The original Portuguese names “Lapa” and “Algar” refer to small and vertical caves, respectively.

Approximately 300 natural cavities are inventoried in Sicó massif (Cunha & Dimuccio 2014). The largest subterranean system of interconnected cave systems in this area is the Dueça Speleological System, that includes the well-known cave of Soprador do Carvalho, which harbours a subterranean river (Neves et al, 2003). Among numerous other exokarst features such as lapies, dry valleys and karsic canyons, approximately 50 sinkholes are found in this region (Cunha 1988). Many of these sinkholes were shaped by locals through time to adapt them into small permanent ponds mainly for irrigation and watering cattle purposes (Cunha & Dimuccio, 2014).

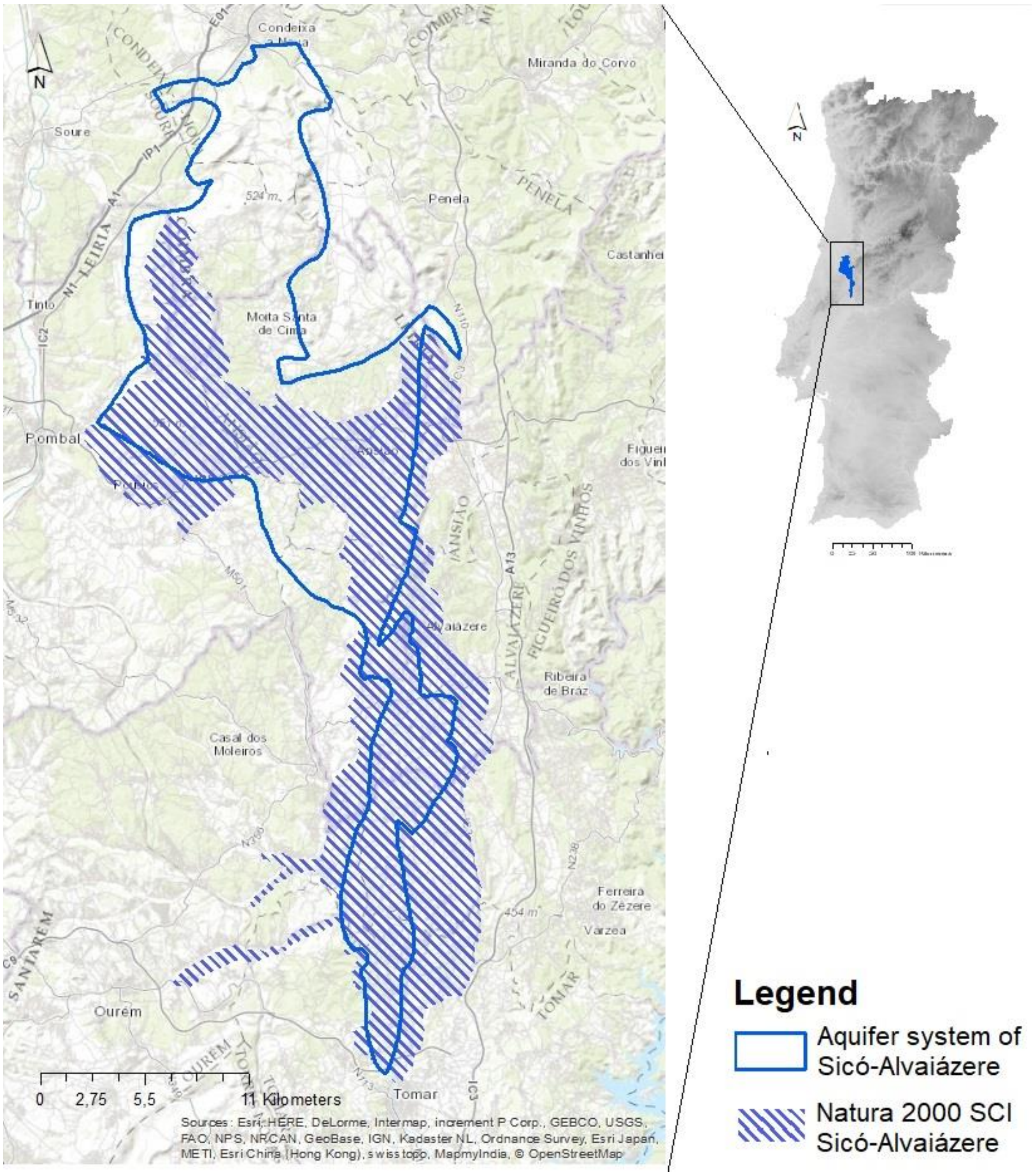

Figure 4. Map of Sicó massif limits (Arcgis).

This massif harbors important and endemic diversity of cavernicolous species (e.g. Pseudoscorpiones and Isopoda) (Reboleira et al 2011; Reboleira et al, 2015). Numerous species of mammals (e.g. *Lutra lutra*, *Miniopterus schreibersii*), fish (e.g. *Rutilus macrolepidotus*, *Lampetra planeri*), plants (e.g. *Narcissus calcicole*, *Juncus valvatus*), amphibians (*Chioglossa lusitanica*) and reptiles (*Lacerta schreiberi* and *Mauremys leprosa*) occurring in this karst are listed in Annex II of Directive 92/43/EEC (ICNF 2017a). Also, important bat refuges that harbor hibernation and maternity colonies are recognized at national level. Regarding amphibians, three species are identified as important: *Rana iberica*, *Pelophylax perezi* and *Triturus marmoratus* (ICNF 2017a). Concerning flora, Sicó-

-Alvaiázere has well-conserved areas of oak (*Quercus faginea* subsp. *broteroi*) and holm oak (*Quercus rotundifolia*) also important endemic species of calcareous flora, such as *Arabis sadina*, *Narcissus calcicola* and *Silene longicilia.* Generally, rock outcrops and cliffs are colonized by casmophitic vegetation, meadows and hills by orchids and succulent plants and limestone gravels which are poor in vegetation. On the Rio Nabão banks, trees like poplars/willows and paludal and riparian forests of alders occur in galleries (ICNF 2017a).

2.2.2. Sampling

The cave selection method for surveying amphibian fauna in natural caves in Sicó massif was based on previous observation record for amphibians and on human accessibility (mainly horizontal or low-depth vertical caves) (Table 1; Fig. 5 and 6).
Horizontal caves (sites 1 to 10) were chosen to assess their use as refuges by amphibian fauna, and vertical caves were also surveyed as to verify the presence of amphibians by accidental falls (site 11, 12 and 13).

The survey was performed in a standard way with the duration of one hour of active search for amphibians in each cave. Search was done mainly close to the entrance area of each cave and then towards its deepest area. A total of seven caves were surveyed in November 2016 (sites 1 to 7), one in December 2016 (site 8) and four in February 2017 (sites 9 to 13). For each location, we registered data on environment variables, namely: surrounding vegetation, occurrence of springs/water bodies nearby, altitude and level of urbanization or isolation of the territory external to the cave. Individuals found were, when possible, captured and measured for biometric parameters (weigh, total body and femur length, diameter) to evaluate their physical condition and biological parameters such as sex and developmental stage. In the impossibility of capture, observation was registered as well. Geographical coordinates of all surveyed locations (caves or water bodies) were collected by Garmin Oregon 550 GPS.

Table 1. List of surveyed caves and geographical information.

Site	Cave	Coordinates	Region	Altitude (m)
1	Gruta da Arrifana	N 39 55,611° W 8 31,059°	Condeixa-a-Nova	145
2	Lapa da Canada I	N 39° 49,298 W 8° 24,940	Alvaiázere	142
3	Gruta da Nascente da Quebrada	N 39,754819° W 8,421175°	Ferreira do Zêzere	143
4	Lapa Grande	N 39,754819° W 8,421175°	Ferreira do Zêzere	143
5	Lapa da Canada II	N 39° 45.299' W 8° 24.927'	Alvaiázere	163
6	Lapa dos Morcegos	N 39,755127° W 8,415665°	Ferreira do Zêzere	138
7	Gruta da Cerâmica	N 39° 55,611 W 8° 31,059	Pombal	346
8	Gruta dos Morcegos	N 39° 39.577’ W 9° 25.074’	Tomar	112
9	Gruta Sta Maria da Estrela	N 39° 59.684' W 8° 32.988'	Pombal	388
10	Algar da Várzea	N 39° 58.730' W 8° 24.135'	Penela	253
11	Algar da Escolabrigo	N 39,936643° W 8,53762°	Pombal	350
12	Algar da Corredoura	N 39° 55.250' W 8° 31.889'	Condeixa-a-Nova	382
13	Algar da Hera	N 39° 55.189’ W 8° 31.952’	Condeixa-a-Nova	401

Gruta da Arrifana, formely known as Cova da Moura, is the major cave of Condeixa-a-Nova region (150m). Next to houses, road (Estrada Nacional nº 1) and a primary school, the cave is accessible through a small entrance facing spring with a lot of surrounding vegetation, including pine trees.

Lapa da Canada I is situated in a relatively isolated area in Alvaiázere, with complex surrounding vegetation, mainly bushes and rock agglomerates.

Gruta da Nascente da Quebrada is a ressurgence of the Olho da Quebrada spring, in Ferreira do Zêzere region, and it was flooded at the time of the survey. Surrounding vegetation cover consists mainly in bushes and trees in a relatively isolated area. Lapa Grande is situated next to Gruta da Nascente da Quebrada, approximately 10 m.

Lapa da Canada II and Lapa dos Morcegos are both situated in Pias and Areias, Ferreira do Zêzere region.

Gruta da Cerâmica is situated in the slope of Monte das Barreirinhas, next to the road to Ereiras, covered by eucalyptus and pine trees and shaded with a dense foliage of bushes. It is considered an important arqueologic site, as several ceramics artifacts and bones have been found in this cave (NEUA, 1985; Santos, 2012).

Gruta dos Morcegos, also known as Gruta do Nabão, is a horizontal cave in a relatively isolated area in Tomar, near the banks of Nabão river. It is an important bat refuge (ICNF, 2017a).

Gruta de Santa Maria da Estrela entrance is situated in a lapies field, close to the geodetic frame of Estrela, in Pombal (NEUA, 1985). It is considered an important bat refuge.

Algar da Várzea, in Penela area, is part of the important and complex speleologic system of Dueça and is the main sink-cave of Sicó-Alvaiázere massif (Neves et al, 2005). The cave can be accessed throught two entrances, althought one was interdict at the time of sampling.

Algar da Escolabrigo is a non-explored small and low-depth vertical cave next to the headquarters of *Grupo de Proteção de Sicó* in Ereiras, Pombal.

Both Algar da Corredoura and Algar da Hera are low-depth vertical caves, located in a relatively isolated area in Condeixa-a-Nova, with a lot of surrounding vegetation (eucalyptus, bushes, etc) and rock outcrops.

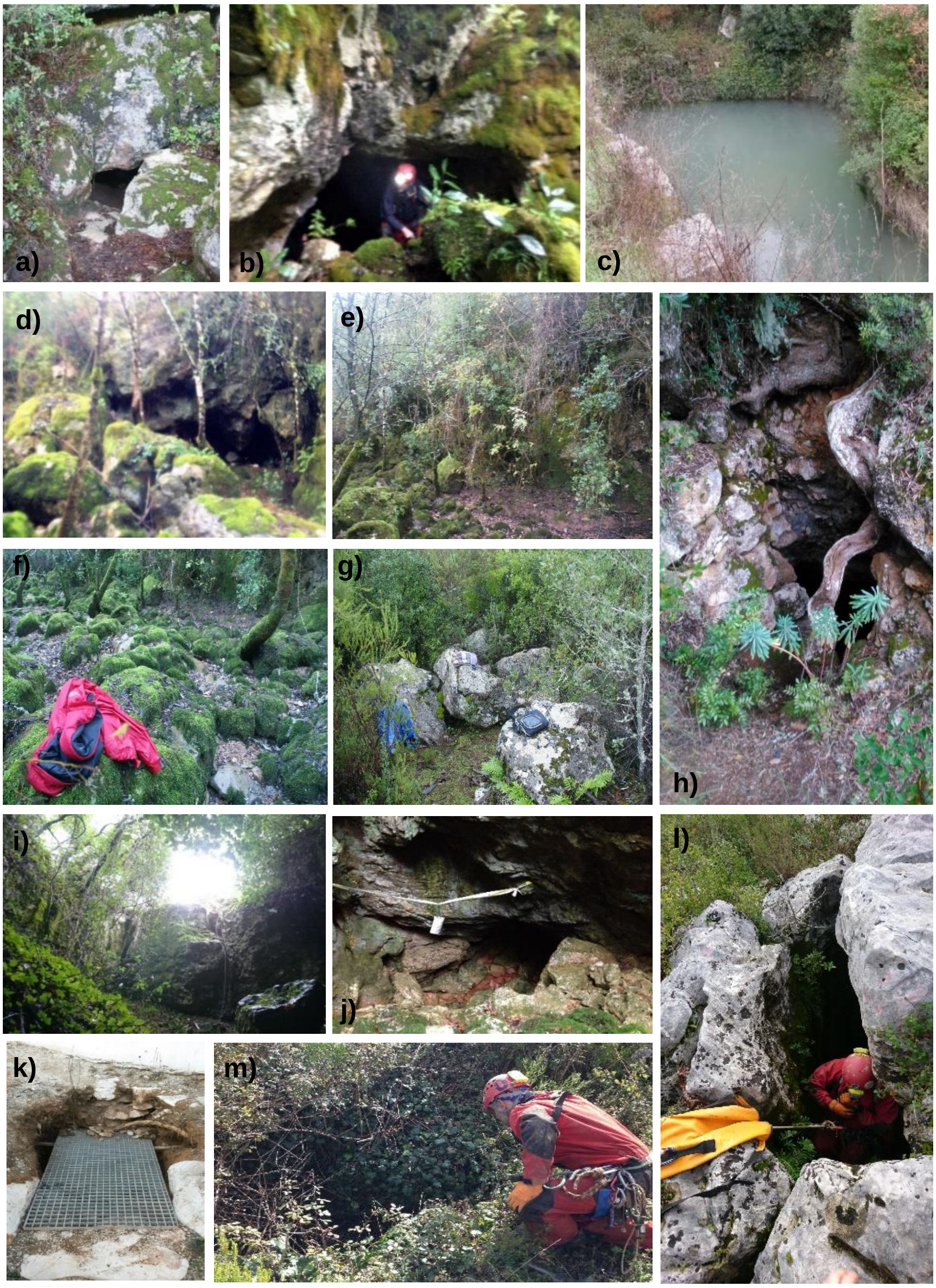

Figure 5. a) Gruta da Arrifana b) Lapa da Canada I c) Gruta da Nascente da Quebrada (source: Geocaching, 2017) d) Lapa Grande e) Lapa da Canada II f) Lapa dos Morcegos g) Gruta da Cerâmica h) Gruta dos Morcegos i) Gruta S Maria da Estrela j) Algar da Várzea k) Algar da Escolabrigo (source: Grupo de Proteção de Sicó) l) Algar da Corredoura m) Algar da Hera.

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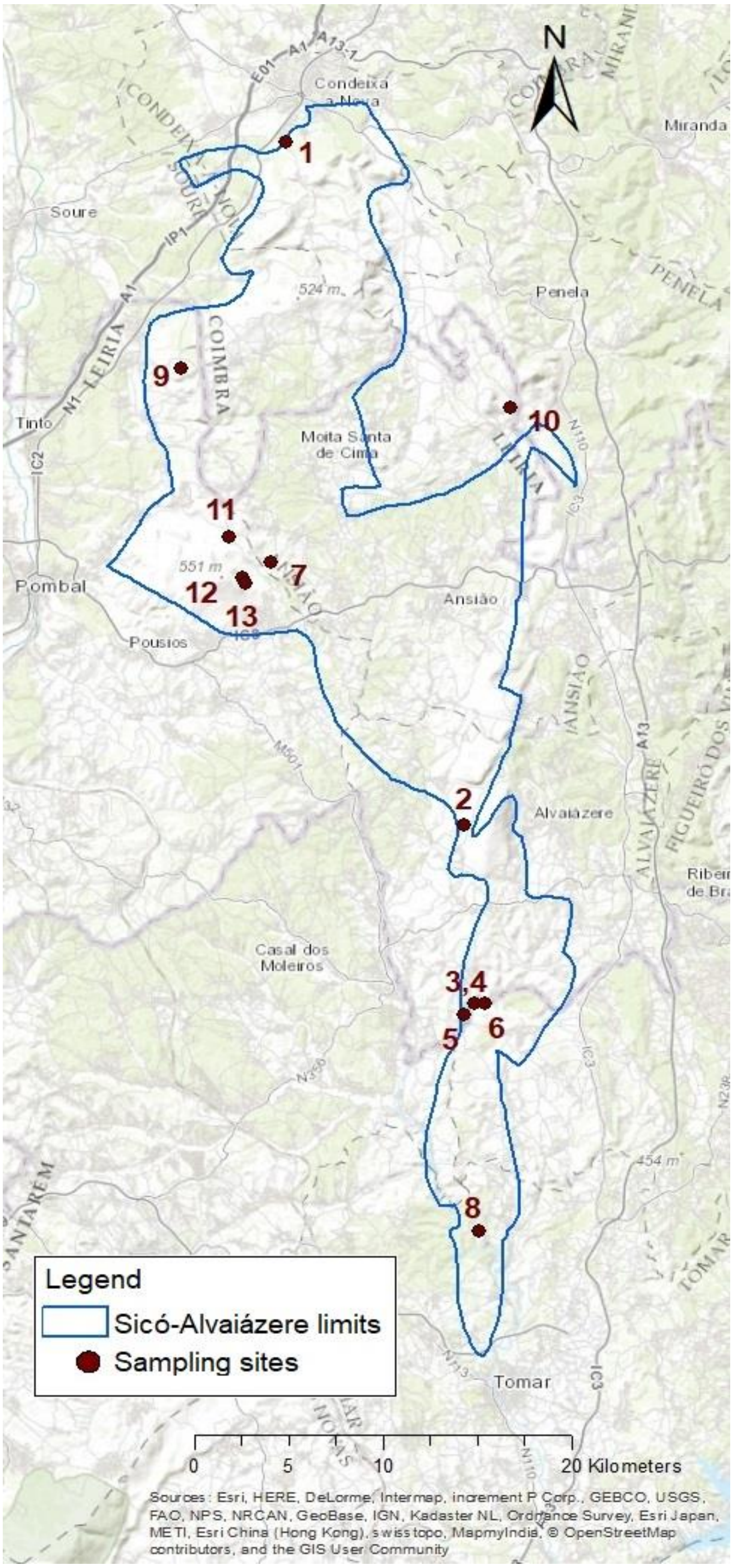

Figure 6. Distribution of the surveyed caves in Sicó Massif (ArcGis).

Map production

Caves surveyed were mapped regarding the presence or absence of amphibian fauna. The maps were generated using ArcMap tool executed in ESRI ArcGIS 10.5 software using the “Natura 2000 Europe” layer and the vector for Sicó-Alvaiázere aquifer system representing the karst boundaries (source: SNIRH 2017).

2.3. Results

A total of four specimens of amphibians were found in three of 13 caves surveyed in this study. Despite the 10 surveyed horizontal caves, only one species (*Triturus marmoratus*) was found (Lapa da Canada, site 2) in November 2016 (Fig. 7). Of the three low-depth vertical caves surveyed, one adult of *Triturus marmoratus* was found in Algar da Corredoura (site 12) (Fig. 8) and two adults of *Bufo bufo* were found in Algar da Escolabrigo (site 11) (Fig. 9) in February 2017. *Bufo bufo* organisms were observed but not captured or photographed. Therefore biometric parameters were only registered for *Triturus marmoratus* specimens (Table 2).

Table 2. Measurements of external morphological traits recorded for each collected specimen (SVL – snout vent length: from snout tip to posterior margin of cloaca; TBL – total body length; BD – body diameter; RLL – rear leg length; FL – femur length; / – impossible to measure due to apparatus malfunction.

Site	Species	Weight (g)	SLV (cm)	TBL (cm)	BD (cm)	RLL (cm)	FL (cm)
2	*T. marmoratus*	/	6.0	11.0	4.0	1.9	1.3
12	*T. marmoratus*	10.5	5.5	10.2	3.9	1.9	0.4

Figure 7. *Triturus marmoratus* found in Lapa da Canada (left); external area of the cave (right).

Figure 8. *Triturus marmoratus* found in Algar da Corredoura (left); external area of the cave (right).

Figure 9. External to the cave Escolabrigo (left); entrance of Algar da Escolabrigo (source: Grupo de Proteção de Sicó) (right).

The map presenting and summarizing the results of the survey, concerning presence or absence of amphibians in caves of the Sicó massif, is shown in figure 10.

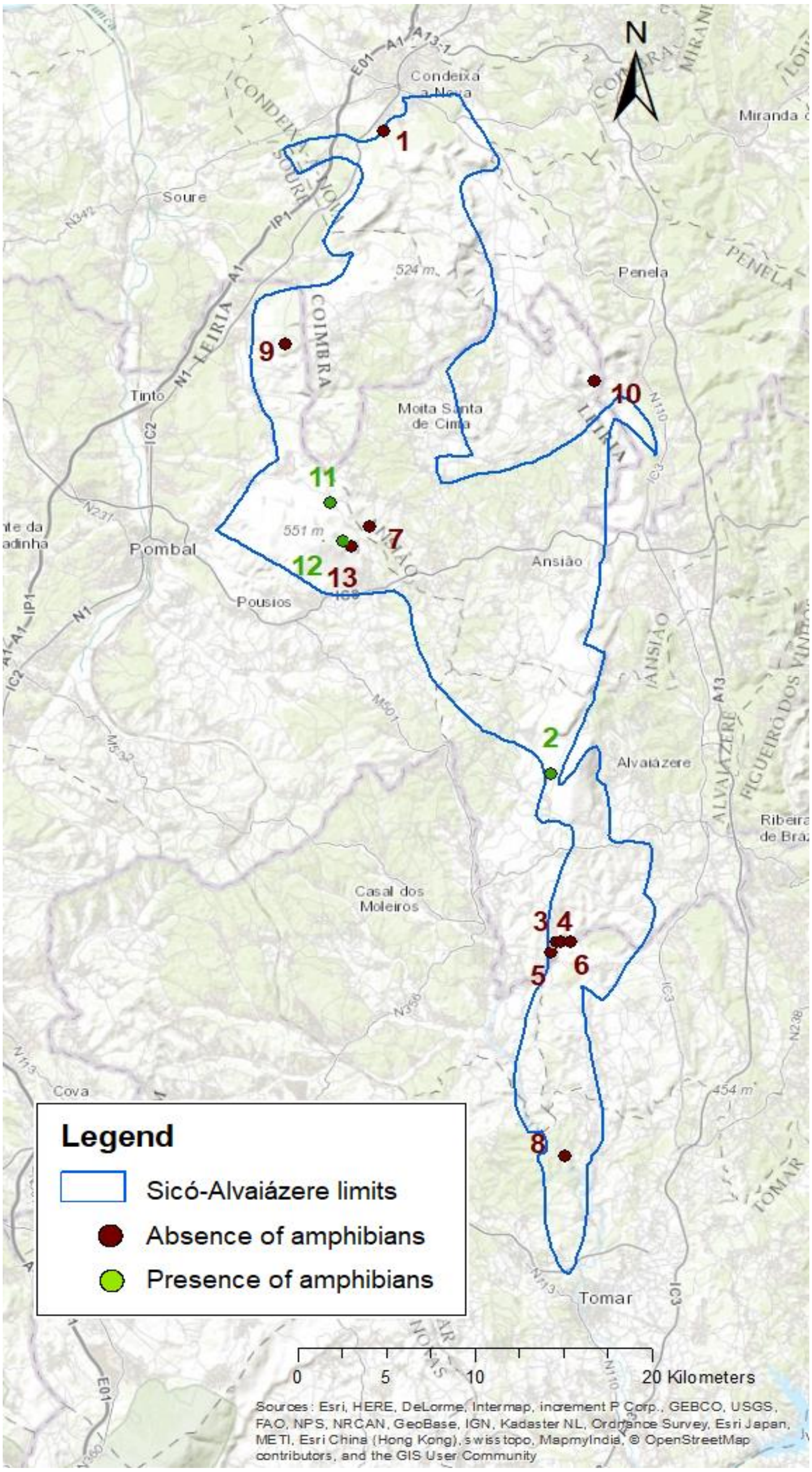

Figure 10. Map illustrating the presence of amphibians in caves of the Sicó massif.

2.4. Discussion

This study contributes with two species to the reduced list of amphibian species found inside caves in mainland of Portugal (see section 2.1): *Triturus marmoratus* and *Bufo bufo*. Both species are commonly found in Sicó massif, the second being listed as an important species in Natura 2000 Sicó-Alvaiázere habitat directive (ICNF, 2017a).

The presence of *Triturus marmoratus* in a horizontal cave (Lapa da Canada I), found a few meters away from the entrance, suggests an active search for this type of habitat. The presence of this specimen's in this cave might suggest that epigean amphibian species may search subterranean habitats as thermal refuge (Resetarits 1986), escaping predators (Manenti et al. 2010; Manenti et al. 2016) or for reproduction (Rosa & Penado 2013). Although no evidence of reproduction was observed, *T. marmoratus* breeding period in central Portugal, lasts from October until May (AmphibiaWeb 2017), coinciding with the sampling period. This species has also been found underground in Spain (Giménez-Lopéz & Guarner Deu 1982).

The presence of another *T. marmoratus* organism in a low-depth vertical cave (Algar da Corredoura) suggests an accidental fall due to the cave's entrance features, surrounded by vegetation, acting as a natural trap. Even though some authors suggest that active exploitation of cave habitats by trogloxene species are very often mistaken for accidental occurrence (Lunghi et al. 2014), this evidence should be supported by additional records.

Overall biometric parameters of both *T. marmoratus* specimens were considered based on visual evaluation and did not allow a distinction between the types of caves in which they were found (horizontal vs vertical).

In spite the presence of *B. bufo*, recorded in the low depth-vertical cave (Algar da Escolabrigo), suggests an accidental fall, this species is known to breed in underground habitats (Bonini et al 1999). Also, they escaped through a very narrow passage inside the cave, making it impossible to take photographic records or biometric parameter measurements. Considering that this cave is unexplored, there is no way to be certain as to where that passage may lead to and thus making conclusions regarding the motif of their presence even more difficult. However, we may take into consideration that these toads were observed in February and that hibernation patterns for this species (occasionally in streams and springs) occurs from September-beginning of November to March-June, while reproduction occurs from March-June (AmphibiaWeb 2012).

2.5. Concluding remarks

We report observation of two amphibian species inside caves of Sicó massif in central continental Portugal: *Triturus marmoratus* and *Bufo bufo*. Amphibians were found in one of ten sampled horizontal caves and in two of four sampled low-depth vertical caves. Presence of amphibians was higher in low-depth vertical type of caves suggesting accidental fall or active search for refuges.

With this study, we hope to trigger further investigation on the presence and importance of subterranean habitats for amphibians and on their ecology in these habitats, in Portugal. Further and regular cave surveys are needed to fully assess and understand the presence of amphibian fauna in Portuguese subterranean habitats. This research shows that the presence of amphibians in caves is underestimated and that more caves should be surveyed for the presence of amphibians in the Sicó massif, but also other main karst areas in Portugal.

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Chapter III

3. Evaluation of the suitability of Sicó massif sinkhole ponds for amphibian early life stages development

keywords Sicó massif, sinkholes, springs, metals, pesticides, amphibians, *Hyla arborea,* biomarkers.

abstract

Karst ecosystems in Portugal are threatened by activities such as agriculture, freshwater supplies, industry, tourism and mining activities, among other sources of pollution and contamination of the surface and consequently the underground. Amphibians (salamanders, toads and frogs) are frequently found in karst environments. Due to the scarcity of water on the surface, sinkhole ponds and springs are essential as breeding sites for these organisms. They are considered to be highly sensitive to a wide range of contaminants, namely pesticides and metals. This study was designed to identify the presence of pesticides in Sicó massif sinkhole ponds and springs and their potential toxic effects on a local aquatic-breeding amphibian species *Hyla arborea*. Here we concurrently evaluate the effects of exposure to water from three sinkhole ponds and three springs on lethal and sub-lethal parameters (oxidative stress biomarkers) on *H. arborea* tadpoles. Water from Dolina de Aljazede had higher influence on the growth and survival rates of tadpoles, although no pattern was established with the biomarkers assessed. The levels of the quantified pesticides were below general reference values, nonetheless the detection of pesticides raises some concerns related to the suitability of the water for amphibians. Further investigation on the potential toxic effects of pesticides and metals on local aquatic breeding amphibian species is required, not only in Sicó massif but also in other portuguese karst areas.

3.1. Introduction

Many economic activities such as agriculture, industry and water exploration, among others (see section 1.1) pose real threats to the integrity and equilibrium of karst landscapes (Watson et al. 1997; Cunha & Vieira 2004; Lee et al. 2012;). The contamination of the groundwater system with toxic compounds such as pesticides, heavy metals, nitrates and other pollutants is mainly caused by agriculture, livestock activities and urban sewage effluents (PBHRO 2011; SNIRH 2017). Based on EPPNA method (qualitative model for assessing aquifer vulnerability based on lithological groups), the two karst systems in Portugal more vulnerable to water contamination are Estremenho and Sicó-Alvaiázere massifs (Pires, 2014).

Sinkholes (or dolines) are common features of karst landscapes, by Field (2002) definition they are “basin - or funnel - shaped hollow in limestone” that when filled with water become sinkhole ponds, which may be temporary or permanent. For humans, these features are important water resources for irrigation and watering cattle purposes, hence agricultural activities are very frequent around these areas. Through time, humans have also shaped these structures as to better suit those needs, namely creating walls around them, adapting the surroundings for accessibility and turning them into permanent ponds. Sinkholes are also extremely important freshwater resources for local fauna since the karst landscape is generally dry on the surface due to inherent infiltration properties of limestones. In such habitats, amphibians are particularly dependent upon these sinkholes.

Due to their biological traits, such as a highly permeable skin, amphibians are generally considered to be highly vulnerable to environmental contaminants released from innumerous anthropogenic activities. Among the various causes responsible for the global amphibian decline (see section 1.2.), exposure to environmental contaminants is one of the most worrisome and generally regarded as a primary factor for this widespread phenomenon (e.g Stuart et al. 2004; Sparling et al. 2010). Heavy metals, fertilizers and pesticides are a few examples from a wide range of pollutants, that can accumulate in the organism’s tissues and potentially be lethal or interrupt/decrease their development, growth and reproduction, among numerous effects (e.g. Kiesecker 2002; Chen et al. 2007; Mann et al. 2009; Ezemonye & Enuneku 2011; Marques et al. 2011; Quarles 2015).

Although still underrepresented in literature, ecotoxicologists have recently and increasingly focused on these organisms to investigate patterns of sensitivity and effects of numerous environmental pollutants (Sparling et al. 2010). Xenobiotics such as pesticides, are highly lipophilic compounds that interact with membrane components potentially causing causing disruption of enzymatic and signal transduction activities, affecting a series of downstream processes and leading to the production of toxic reactive oxygen species (ROS) (Venturino et al, 2003), which can lead to severe cellular damage and diseases (Boelsterli 2007). In fact, oxidative stress has been emphasized as a common consequence underlying the toxic action of most xenobiotics (e.g. Limón-Pacheco & Gonsebatt 2009; Boelsterli 2007). Several approaches employ the use of molecular biomarkers because they can establish a link between environmental contamination and effects on organisms, allowing an accurate assessment of sub lethal endpoints of intoxication, long-term effects, bioavailability and interactions between multiple toxic chemicals, among others (see Pechen de D'Angelo & Venturino 2005; Venturino et al, 2003). A specific antioxidant system – reduced glutathione (GSH) - and associated enzymes: glutathione S-transferase (GST) and glutathione peroxidase (GPx) have been highlighted as overall strong biomarkers of contaminant-induced oxidative stress (Isaksson 2010; Venturino et al. 2003a). Byproducts of lipid peroxidation, which is a consequence of the ROS-induced damage, such as thiobarbituric acid reactive substances (TBARS) are also considered to be biomarkers of oxidative stress (Pechen de D'Angelo & Venturino 2005). These biomarkers have been widely used in amphibian ecotoxicology generally regarding heavy metals and pesticide's effects (e.g. Venturino et al, 2001; Marques et al, 2011).

This study was designed to identify the presence of pesticides in Sicó massif sinkhole ponds and their potential toxic effects on amphibian species. This study concurrently evaluates the effects of exposure to water samples from these potential breeding ponds on lethal and sub-lethal effects (oxidative stress biomarkers) on *Hyla arborea* tadpoles

3.2. Material and methods

3.2.1. Study site

Sicó massif is considered one of the main karst areas in the Orla Mesocenozóica Ocidental, in central Portugal, occupying an area of about 430 km^2 (Vieira & Cunha 2006). It is classified as Site of Community Importance (SCI) integrated in Natura 2000, since 2008 (ICNF, 2017). Under the influence of an Atlantic climate, this massif covers a set of relatively low hills, mountains (Sicó: 553 m and Alvaiázere: 618 m), uplands and other diverse limestone associated habitats covering Alvaiázere, Ansião, Condeixa-a-Nova, Penela, Pombal and Soure counties (Vieira & Cunha 2006).

Among numerous other karst features considered as patrimonial elements of Sicó massif, such as caves, lapies fields with sparse vegetation (referred as “rock deserts”) and fluvial karstic canyons, exsurgencies and sinkholes are particularly important as water resources for public supply (Cunha & Vieira 2004).

The complex subterranean system of Dueça is a set of exsurgencies and a few sink-caves (Algar da Várzea) conected by a series of cavities, such as Soprador do Carvalho that is known for harbouring a subterranean river (Neves et al, 2003). On the western part of the massif, water discharge is set through some temporary but mostly permanent springs, such as Ourão, Arrifana and Olhos de Água do Anços. The latter being the most important where about 60% of circulation waters reemerge. On the eastern part, the main springs, such as Olhos de Água do Dueça, are temporary or seasonal (Cunha & Dimiuccio 2014). Concerning sinkholes, approximately 50 are found in this region (Cunha 1988). Many of these sinkholes were shaped by locals through time to adapt them into small permanent ponds mainly for irrigation and watering cattle purposes (Cunha & Dimuccio, 2014).

A total of six sinkholes were sampled in the northern part of the massif (Table 3 and fig. 11). Geographical coordinates of each sample site were taken with AQUAREAD multiparametric probe (Aquaread AP-2000; gps Garmin 550).

Table 3. List of sampled sites and geographical information.

Site	Sinkhole name	Coordinates	Altitude (m)
1	Nascente do Ourão	N 40,0267° W 8,5897°	50
2	Nascente de Anços	N 39,9864° W 8,5769°	75
3	Dolina dos Casais de São Jorge	N 40,0111° W 8,5386°	301
4	Dolina de Aljazede	N 39,9803° W 8,42°	271
5	Olho do Dueça (Taliscas)	N 39,9994° W 8,3914°	196
6	Dolina da Confraria	N 39,9422° W 8,5328°	435

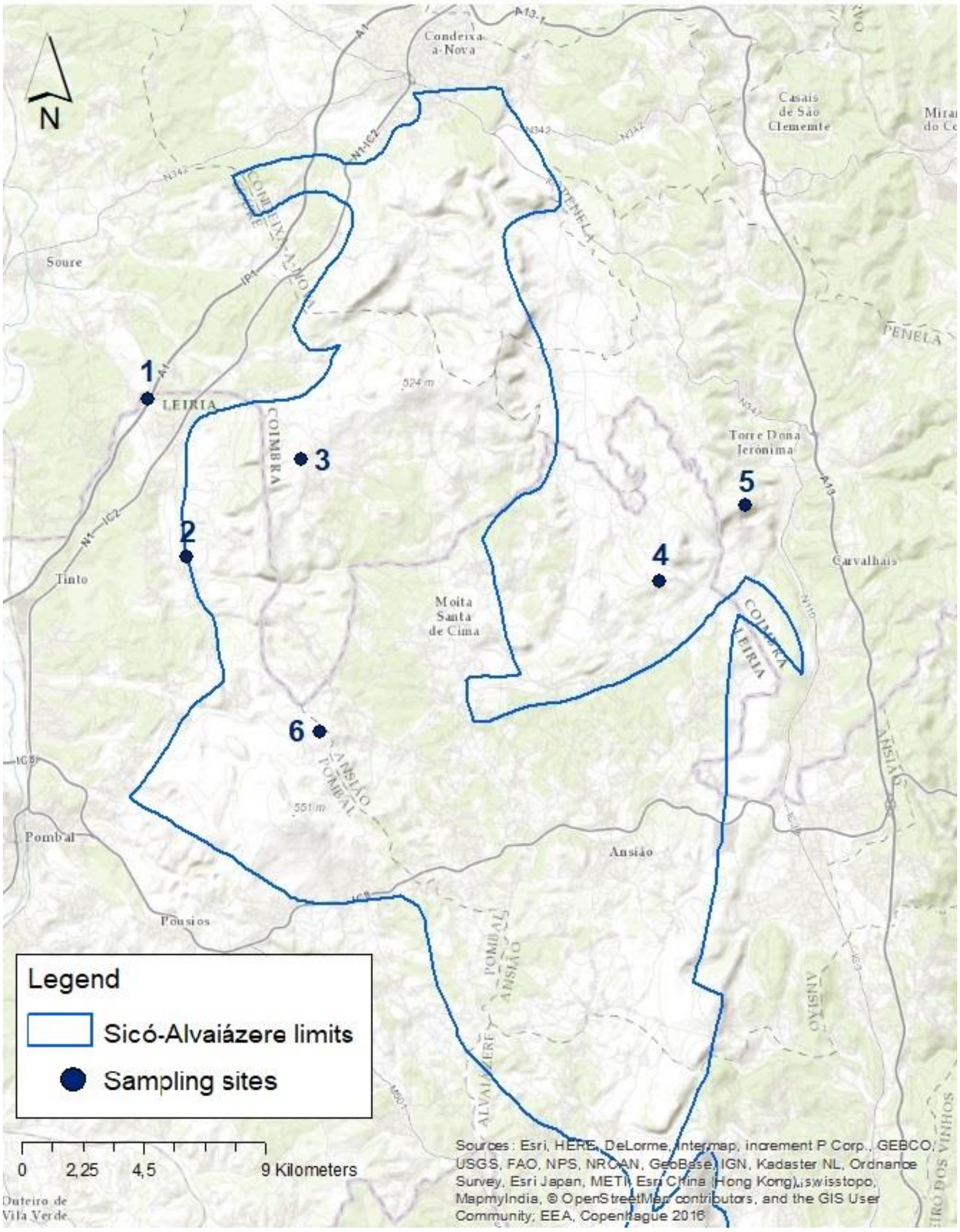

Figura 11. Distribution of sampled sinkholes in northern Sicó Massif (blue limit represents Natura2000 protected site: Sicó-Alvaiázere): 1. Nascente de Ourão, 2. Nascente de Anços, 3. Dolina Casais de São Jorge, 4. Dolina de Aljazede, 5. Olho do Dueça and 6. Dolina da Confraria (ArcGis).

From direct observation of the sampled sinkholes, we could infer that the majority were impacted and eutrophic to some degree and most were currently inappropriate for human consumption, as local population highlighted it. Agricultural activities and/or cattle were seen near most locations; hence it is fair to assume some level of water contamination from pesticides or fertilizers through rainfall and runoffs. One of the sampled sites is a documented case of high contamination of subterranean water in Sicó Massif draining at the spring of the Anços river, which is considered dangerous and bacteriologically inappropriate for human consumption (Reboleira 2005).

The first sampled site, Nascente do Ourão (Fig. 12), is a permanent and artificial pond, limited by a stone wall and situated in a former quarry that belonged to the Ourão farm located in Barreiras, Pombal (NEUA 1985). Here is the spring of the Ourão river, with the Ourão Cave located in the margin. This spring is the major public water supply of Pombal region (CMP 2014). It is surrounded by houses, roads and vegetation, probably being a breeding site for some amphibian species, since numerous *Pelophylax perezi* organisms were observed during sampling.

Nascente de Anços (Fig. 13) is the spring of the Anços river. It is the main permanent spring of the region – Olhos d'Água (NEUA 1985). It is situated in the middle of Redinha parish, surrounded by roads, houses and vegetation.

Figure 12. Sampling site 1 – Nascente do Ourão

Figure 13. Sampling site 2 – Nascente de Anços.

Dolina dos Casais de São Jorge (Fig. 14) is a small sinkhole pond next to a road, houses, a company of dried fruits production and vegetation, temporary filled with water. The water of this sinkhole appeared to be somewhat impacted and litter was found at the margins. During sampling, many organisms of *Pelophylax perezi* were observed, suggesting that this can be a reproduction site for amphibians. Also we found a snake (*Natrix maura*) and some crayfish (*Procambarus clarkia*) were found in this pond.

Figure 14. Sampling site 3 – Dolina dos Casais de São Jorge.

Dolina de Aljazede (Fig. 15) is a small temporary pond surrounded by agricultural lands, forest and roads. Although no amphibians were observed, vocalizations indicated their presence in this sinkhole.

Figure 15. Sampling site 4 – Dolina de Aljazede.

Olho do Dueça (Fig.16) is an artificial well located between agricultural lands and next to houses and roads. It is the spring of the river Dueça and is part of the speleological system of Dueça, considered to be one of the most important and complex cave systems of continental Portugal, integrating 15 caves and two sinkholes (Dolina de Taliscas and Dolina do Sobral) (NEUA 2005). It is limited by a stone wall and surrounding vegetation. *Pelophylax perezi* organisms were also observed in this site.

Figure 16. Sampling site 5 – Olho do Dueça.

The last sampling site, Dolina da Confraria (Fig. 17), is situated in a relatively isolated area, surrounded by roads and vegetation. In this pond, one adult and three juveniles of *Salamandra salamandra* and *Triturus marmoratus* larvae were observed, evidence that this is an amphibian breeding site.

Figure 17. Sampling site 6 – Dolina da Confraria.

3.2.2. Water sampling

Sampling of all six sites was performed in May 4, 2017. Conductivity, dissolved oxygen (percentage and mg/L) and pH values were measured on each site before water sampling using AQUAREAD multiparametric probe (Aquaread AP-2000; gps Garmin 550). Water samples for the bioassays were collected in 6L plastic containers and stored at 4ºC in a refrigerated chamber throughout the duration of the bioassays. For pesticide analyses, water samples were collected in 1.5 L plastic containers and stored at -20ºC until analyses were possible.

3.2.3. Chemical analysis

The quantification of pesticides for the collected water and also the FETAX control was conducted by EUROFINS LaboratoryThe pesticides selected and the analytical method were: terbutylazine, tebuconazole, dimethomorph and glyphosate through LC/MS/MS and deltamethrin and chlorpyrifos through GC/MS.

Evaluation of other parameters such as total hardness and alkalinity also ammonium, nitrites and orthophosphate contents were measured for each water sample with Aqualytic PC multidirect photometer system following the manufacturer's instructions.

3.2.4. Test organisms

The present study was conducted under the supervision of an accredited expert in laboratory animal science (following Federation of Laboratory Animal Science Associations (FELASA) category C recommendations) and according to the European guidelines on protection of animals used for scientific purposes (directive 2010/63/UE of European Parliament and the Council of European Union).

Hyla arborea (Linnaeus, 1758) was selected to be the test organism because it is a representative species of the amphibian fauna occurring on Sicó massif (Oliveira & Pargana 2008) using preferably ponds, lakes and reservoirs for reproduction. This species is listed with a least concern conservation status in Portugal (Oliveira & Pargana 2008). However, it has been highlighted that some local populations might be threatened due to increasing urbanization and agriculture, destruction of vegetation and contamination of water bodies, among others (Loureiro et al. 2008) making this a species of particular interest for conducting exposure assays with water from potential breeding ponds, at a regional level.

3.2.5. Bioassay

On May 13, 2017, clutches of fertilized *Hyla arborea* eggs were collected from a temporary pond in Espinhel, Aveiro (N 40º34.335 W 8º29) and were transported to the laboratory at Universidade de Aveiro (Aveiro, Portugal) in plastic containers (Fig. 18). Viable eggs between developmental stages 9-12 (Gosner 1960) were selected and placed into glass vessels with FETAX medium (Dawson and Bantle, 1987). The eggs were then arbitrarily placed into glass vials (200 mL) filled with 150 mL of each water from each site and control with FETAX medium. Each water treatment (6 locations) was replicated 5 times (20 eggs per replicate). Every other day the exposure media and FETAX control were renewed and on a daily basis mortality was checked and dead animals removed. Throughout the exposure animals were maintained under contant temperature (20ºC) and photoperiod (16hL:8hD) (Fig. 19-20). The bioassay ended when all the FETAX control animals reached the 25th Gosner development stage (Gosner 1960). Dissolved oxygen, pH and conductivity were measured with Aquaread AP-2000 portable multiparametric probe at the beginning of the essay and again at the end. At the end of the assay the animals were assessed for abnormalities and 10 tadpoles per replicate were randomly selected to be measured under OLYMPUS SZX9 macro zoom stereoscope.

Figure 18. Temporary pond where *H. arborea* egg masses were collected (left); *H. arborea* egg masses (right).

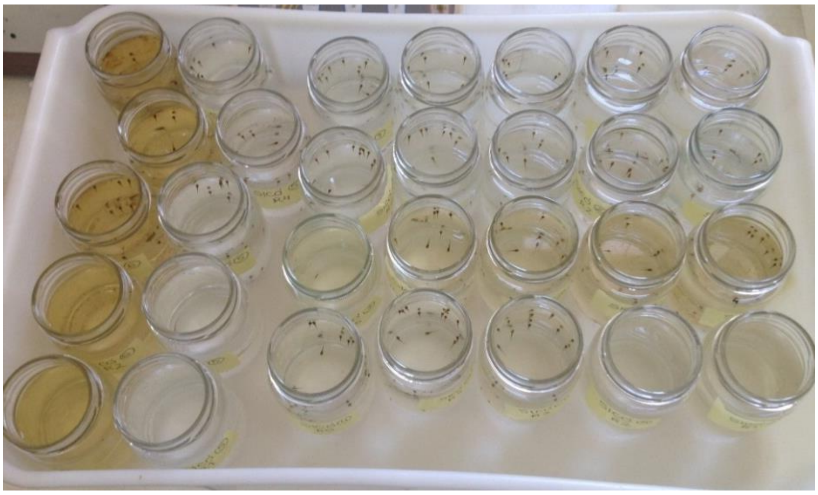

Figure 19. *H. arborea* bioassay: exposure to water samples collected from each site.

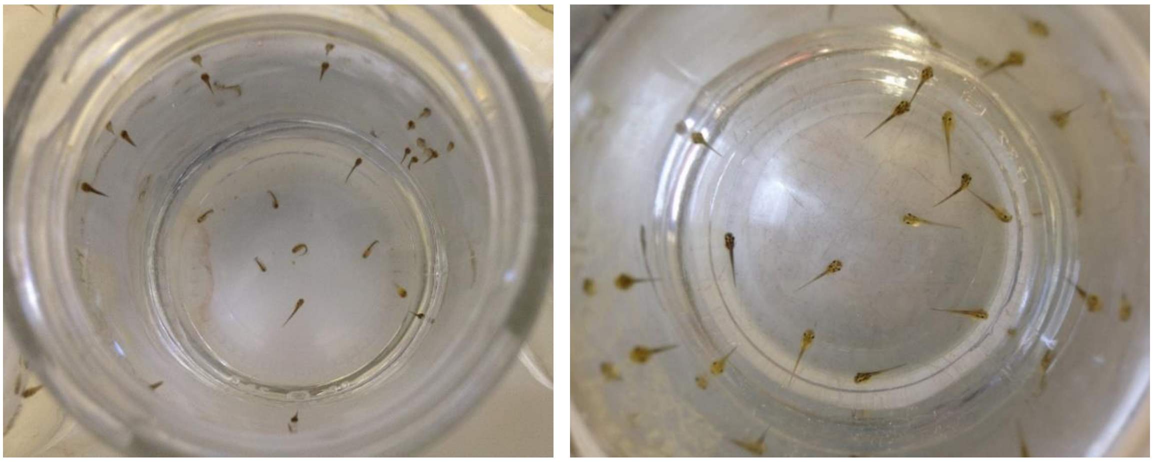

Figure 20. Bioassay with *H. arborea* on the 7th day (left) and on the 11th day of exposure (right).

3.2.6. Oxidative stress biomarkers

Oxidative stress biomarkers were evaluated in the total mass of tadpoles from each replica through enzymatic determination of GSTs, GRed and total and selenium-dependent GPx. Also, the measurement of thiobarbituric acid reactive substances (TBARs) allowed the assessment of lipid peroxidation.

Tadpoles from each replica were previously frozen in liquid nitrogen and homogenized in phosphate buffer (50 mM, pH= 7.0 with 0.1 % TRITON X-100). Homogenates were then centrifuged at 10,000 g for 10 min and supernatants were divided into 5 eppendorfs - one for each determination (GPx, GST, GRed, TBARs and protein) - and stored at -80 ºC until determination was possible.

GSTs (EC 2.5.1.18) activity was measured by spectrophotometry, following Habig et al. (1974) protocol. The formation of thioeter (molar extinction coefficient of 9.6 mM^{-1} cm^{-1}), resultant from the conjugation of the substrate chloro-2,4-dinitrobenzene (CDNB) with glutathione, was followed with an absorbance of 340 nm.

GRed (EC 1.8.1.7) activity was measured by spectrophotometry, following Carlberg and Mannervik (1985) protocol. The oxidation of NADPH (molar extinction coefficient of 6.22 $mM^{-1}cm^{-1}$), mediated by this enzyme, was determined at a wavelength of 340 nm.

GPx (EC 1.11.1.9) activity was measured by spectrophotometry, following Flohé and Günzler (1984) protocol. The oxidation of NADPH (molar extinction coefficient of 6.22 $mM^{-1}cm^{-1}$), occurring when glutathione reductase catalyzes the reduction of GSSG into GSH, GPx activity was determined at a wavelength of 340 nm, using independent substrates: cumene hydroperoxide (0.7 mM) corresponding to total glutathione peroxidase, hydrogen peroxide (0.255 mM) corresponding to selenium-dependent glutathione peroxidase.

Enzymatic activities were expressed in nmol (GSTs, selenium-dependent GPx and total GPx) or µmol (GRed) of substrate hydrolyzed per min per mg of sample protein.

The quantification of TBARS (molar extinction coefficient of 1.56x105 $M^{-1}cm^{-1}$) was determined spectrophotometrically, according to Buege and Aust (1978) described protocol, at a wavelength of 535 nm. The reaction is determined by lipid peroxidation by-product - malondialdehyde (MDA) - with 2-thiobarbituric acid (TBA). The results were expressed as nmol of MDA equivalents per mg of sample protein.

Protein concentration was determined for each replica by spectrophotometry at a wavelength of 595 nm, according to Bradford (1976) method (adapted to microplates). As mentioned above, the determination of all biomarkers (GSTs, GRed, total and selenium-dependent and TBARS) were expressed in function of the protein content of the corresponding sample.

3.2.7. Statistical analysis

Before testing, all data were checked for normality and homogeneity to meet statistical demands and when needed, data transformations were applied (lg10 or ln gamma). To test for statistical significant differences between organisms exposed to different treatments, for each enzyme activity (GST, GRed, total GPx and GPx selenium-dependent) and TBARS and also significant differences between tadpoles' length on the last day of the assays. Data was analyzed through parametric one-way analysis of variance (ANOVA) followed by a post hoc multiple comparison Tukey´s test. To reject the null hypothesis, a level of significance of 0.05 was chosen.

3.3. Results

Water samples chemical characterization

Water parameters such as hardness, alkalinity and ammonium, nitrites and phosphate concentrations as well as pH, conductivity, dissolved O_2, salinity and total dissolved solids (TDS) for all six sites and FETAX medium are presented in table 4.

Total alkalinity and total hardness were both higher in site 5 and lower in sites 3 and 6 respectively. Ammonium concentrations ranged from <0.02-0.29 mg/l in sites 2 and 5 respectively. Nitrites quantification determined values ranging from <0.01-0.04 mg/l, with all sites except 5 presenting the minimum value. While phosphates ranged from <0.05-0.12, the latter in sites 3 and 5.

Values of pH in all sites ranged from 7.11-7-65 with the highest value in site 3;

Conductivity from 154-499 µS/cm, the latter measured in site 5 and the highest value found in control (899); Dissolved oxygen saturation ranged from 94.3-98.5%, the latter measured in site 5; Salinity ranged from 0.03-0.22 PSU, the minimum value occured in site 3 and the maximum in both sites 1 and 2; TDS ranged from 70-351, site 3 and 5 respectively.

Table 4. Water samples and FETAX chemical characterization.

	REF	**Control**	**1**	**2**	**3**	**4**	**5**	**6**
Hardness, total ($CACO_3$) (mg/l)	-	460	42	65	24	28	115	80
Alkalinity, total ($CACO_3$) (mg/l)	2000	82	200	190	220	160	310	135
Ammonium (NH_4^+) (mg/l)	-	0.10	0.21	<0.02	0.09	0.11	0.29	0.07
Nitrite (NO_2^-) (mg/l)	-	<0.01	<0.01	<0.01	<0.01	<0.01	0.04	<0.01
Phosphate (PO_4) (mg/l)	-	0.11	<0.05	<0.05	0.12	<0.05	0.12	<0.05
pH	6.0-7.5	7.56	7.10	7.11	7.65	7.36	7.20	7.29
Conductivity (µS/cm)	150-500	899	458	407	154	223	499	296
O_2 %	≤60	96.00	95.50	94.28	95.85	94.33	98.53	92.40
O_2 (mg/l)	-	8.45	9.16	9.06	9.00	8.72	9.25	8.64
Salinity (PSU)	-	-	0.22	0.22	0.03	0.08	0.23	0.10
TDS	50-250	-	347	345	70	166	351	202

REF stands for Reference values for aquatic life (Dodd 2010).

The analysis and quantification of pesticides for all six sites (Table 5) was compared with the legally established values and values for LC50 for both freshwater fish and amphibian model species, due to lack of some values for the latter.

Pesticides (µg/l)	MAV	FWG	LC50 FISH	LC50 AMP	EQG	FETAX	Site 1	Site 2	Site 3	Site 4	Site 5	SITE 6
Terbutylazine		NLE	1800	-	NLE	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005
Tebuconazole		NLE	1135	-	NLE	<0.005	<0.005	<0.005	<0.005	<0.023	<0.005	<0.005
Dimethomorph	0.5	NLE	3100	-	NLE	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005
Deltamethrin		0.0004	0.9	7.1	NLE	<0.16	<0.16	<0.16	<0.16	<0.16	<0.16	<0.16
Chlorpyrifos-ethyl		0.0035	0.29	>400	0.1	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Glyphosate		65	21500	>2600	NLE	<0.02	<0.02	<0.02	4.1	0.028	<0.02	<0.02

Table 5. Pesticide quantification data (µg/l) for FETAX and sites 1-6, comparative legal values and LC50 for freshwater fish (EPA 2017) and amphibians (Crane et al. 2016).

MAV stands for Maximum admissible value for environmental water quality in Portugal (per individual pesticide substance) (MA 1998).

FWG stands for Fresh Water Guidelines retrieved from PAN pesticides database and representing Canadian water quality guidelines for the protection of fresh water aquatic life (PAN 2017).

NLE stands for No Legal Values established.

LC50 stands for Lethal Concentration that is lethal for 50% of the population.

EQG stands for Environmental Quality Guidelines – values for maximum admissible concentrations for Europe (Directiva 2008/105/CE).

Exposure assays

Mortality occurred in all exposures, except for control (Table 6). At the end of the essay, higher rates of mortality were verified among sites 4 and 6. The average mortality for sites 1 to 6 was 4, 5, 3, 9, 3 and 8% respectively. Malformations occurred (Fig. 22), nonetheless the number of cases were negligible. Statistical analysis of body length measurements (Fig. 21) revealed diferences between organisms exposed to water from site 4 and from sites 1,3,5,6 and control ($F = 5.968$; $df = 6$; $p <0.01$) with organisms from site 4 presenting a lower body size.

Table 6. Results from exposure assays: mortality and anomalous tadpoles.

Replicates	Control	Site 1	Site 2	Site 3	Site 4	Site 5	Site 6
R1	20	16	20	20	20	19	19
R2	20	20	17	19	17	20	20
R3	20	20	19	20	17	18	19
R4	20	20	19	20	20	20	18
R5	20	20	20	18	17	20	16
Mortality (%)	0	4	5	3	9	3	8
Anomalous (%)	4	0	0	0	0	1	1

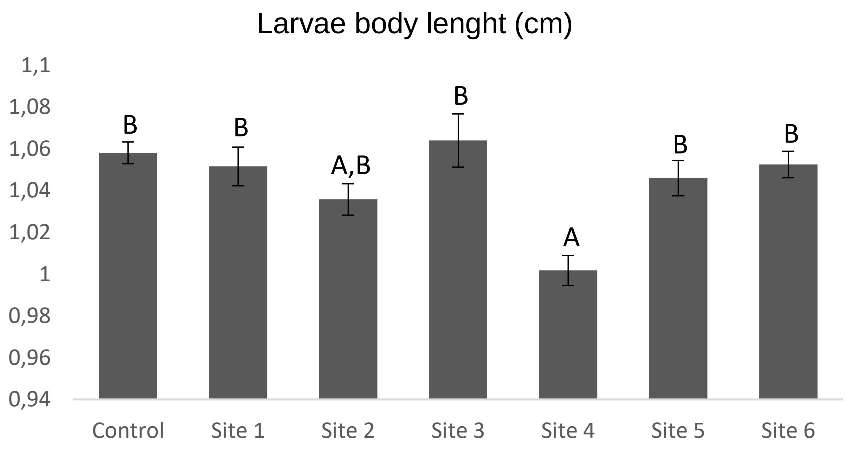

Figure 21. Larvae body length after the 11-day exposure assay. A and B represent statistically significant different groups ($p < 0.05$).

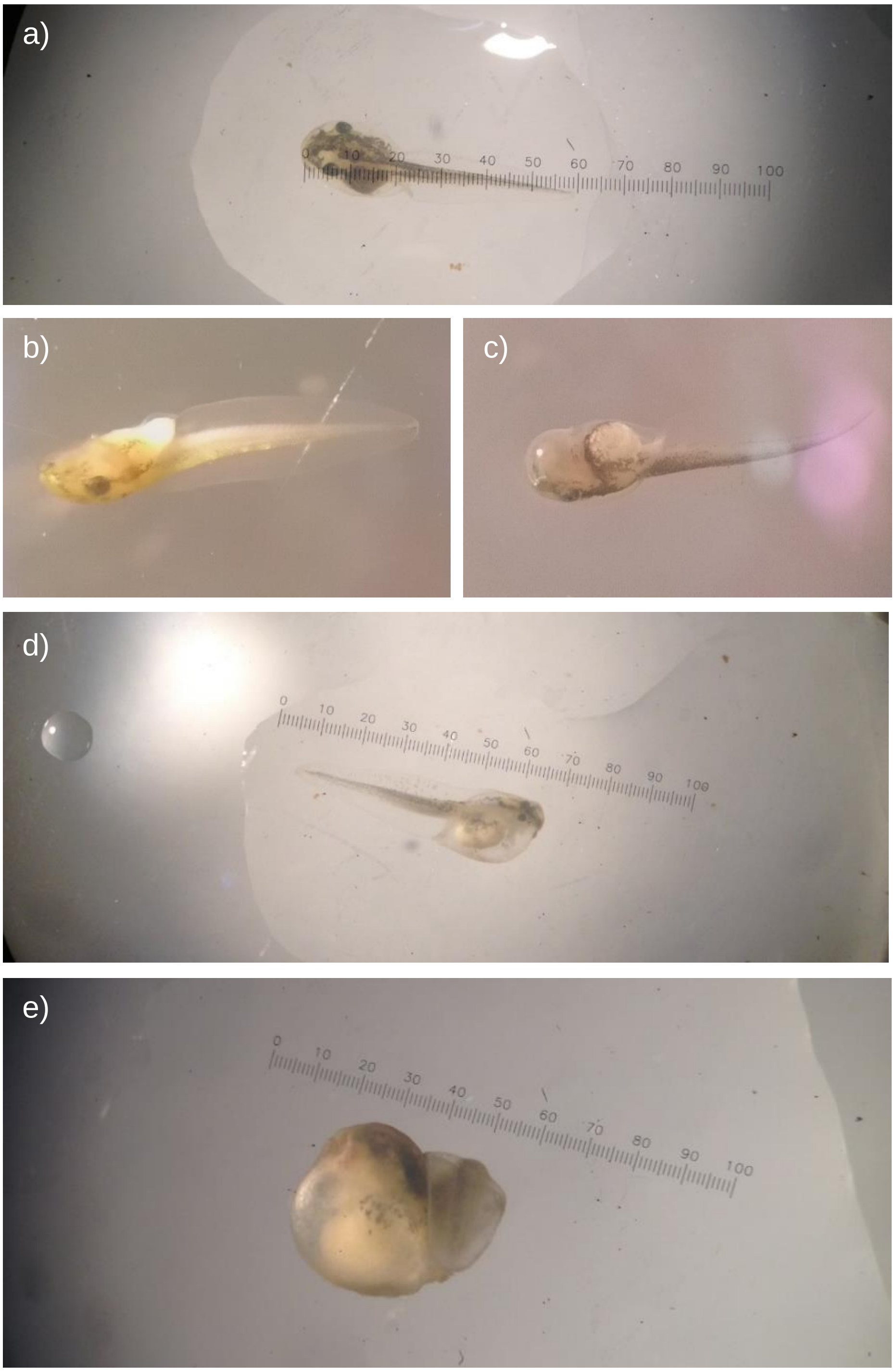

Figure 22. a) Example of *Hyla arborea* tadpole with normal development; b), c), d) and e) are examples of the different malformations observed.

Biomarkers

The statistical analysis of the biochemical data showed no statistical differences between factors (sites 1 to 6 and control) for GSTs, GRed, GPx peroxide and total GPx (Fig. 23). Statistically, the only significant diferences between sites were found in lipid peroxidation between organisms exposed to water from site 1 and those exposed to waters from sites 4, 5 and 6 ($F = 270$; $df = 6$; $p < 0.01$) (Fig. 25).

GSTs activity was higher in sites 6 and 4, followed by sites 2, 1 and control whilst lower in sites 5 and 3 - which had also the lowest rates of mortality (3%) - pattern that was followed for all other enzymes. GST for both these sites (5 and 3) was even lower than the results from individuals from control. GRed activity was higher in sites 4, 1 and 2 and the lowest in sites 6 and 5, respectively. Se-dependent GPx activity was higher in control and sites 1 and 6 and the lowest in site 5. In turn, total GPx activity was higher in sites 1, 2 and control respectively, and the lowest in sites 5 and 3.

TBARS quantification was higher in sites 6 and 3 and the lowest in sites 1, 2 and control, respectively.

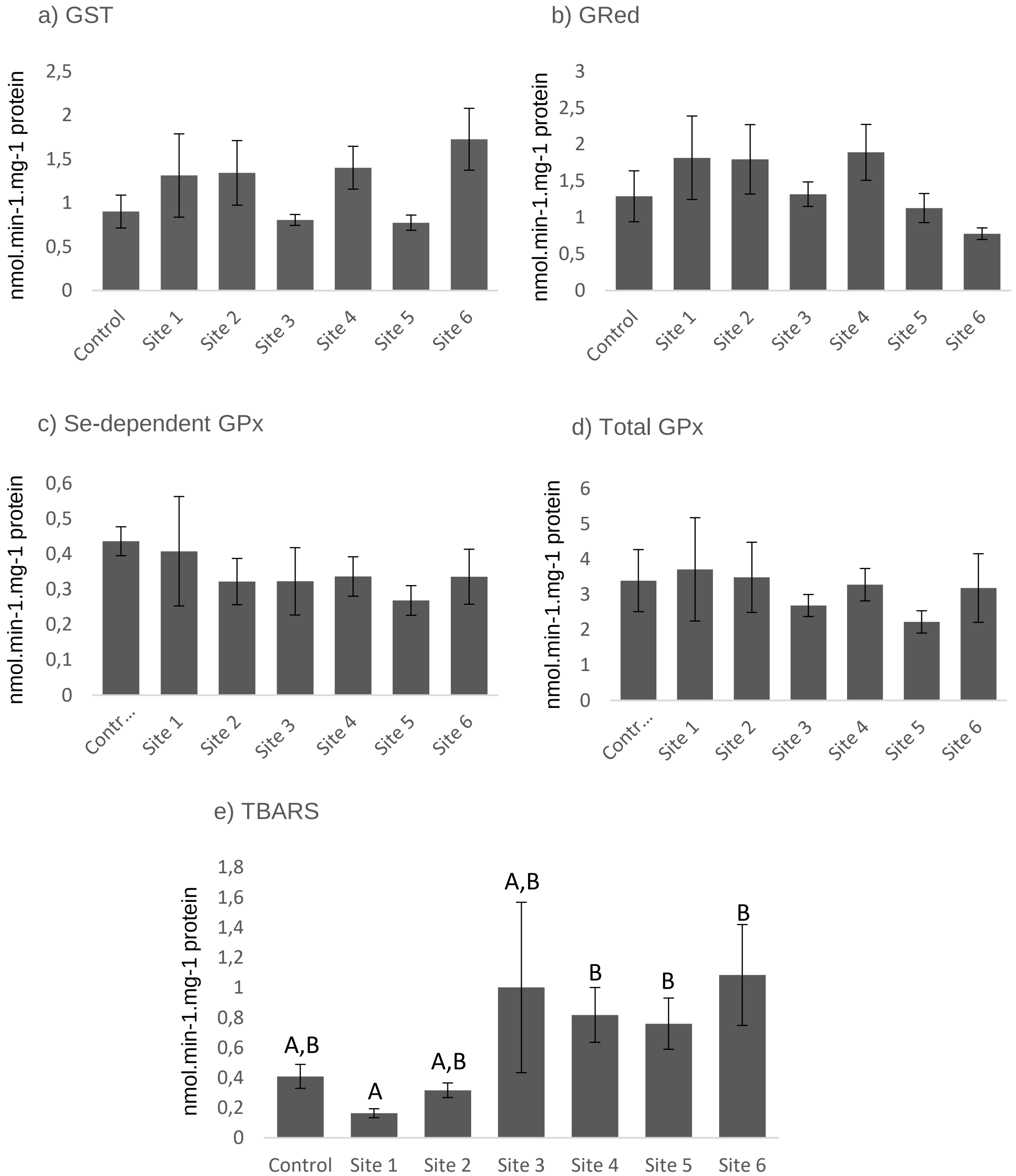

Figure 23. a) Mean GSTs activity in *H. arborea* tadpoles after 12-day exposure to water samples from sites 1-6 and respective control. b) Mean GRed activity in *H. arborea* tadpoles after 12-day exposure to water samples from sites 1-6 and respective control. c) Mean Se-dependent GPx activity in *H. arborea* tadpoles after 12-day exposure to water samples from sites 1-6 and respective control. d) Mean total GPx activity in *H. arborea* tadpoles after 12-day exposure to water samples from sites 1-6 and respective control. e) Mean TBARS activity in *H. arborea* tadpoles after 12-day exposure to water samples from sites 1-6 and respective control. Error bars represent standard error. Statistical analysis was performed within exposure sites and control. A and B: represent statistically significant different groups ($p < 0.05$).

3.4. Discussion

Poor water quality and contaminants such as pesticides and metals are a great threat to amphibians (Stuart et al. 2004; Sparling et al. 2010) particularly during the initial stages of their life cycles, where they are usually confined to stagnant water bodies that easily accumulate toxic substances, particularly near agricultural areas. The consequences can be lethal or sub-lethal effects such as growth, development, reproductive and behavioural impairments (e.g. García-Muñoz et al. 2009; Ezemonye & Ilechie 2010; Marques et al. 2011).

In this study, *H. arborea* tadpoles exposed to water collected from Dolina de Aljazede presented overall smaller body sizes, while control and the majority of the tadpoles exposed to other conditions showed larger body sizes. However, there were no significant diferences between them. The effect of body length reduction is suggested to be a consequence of energy deviation to detoxification pathways (Wright and Wright 1996 in García-Munoz et al 2011). Also, this reduction in size has been correlated with an increase in developmental time (Breden and Kelly 1982) which for certain species, that breed in temporary ponds, can signify that some individuals may not reach metamorphosis in time before the ponds dry. Although no chemical parameter evaluated stood out regarding site Dolina de Aljazede, pesticides quantification revealed the presence of gliphosate even though it was inferior to the legally established safety values for aquatic life as well as LC50 values for both fish and amphibians.

Mortality rates were also higher in Dolina de Aljazede (9%), followed by Dolina da Confraria, Nascente de Anços, Nascente de Ourão, Dolina dos Casais de São Jorge and Olho do Dueça. This results also stress out that there might be some unidentified contaminant or stressor in Doluna de Aljazede, causing these effects.

Regarding chemical parameters, sampled waters generally presented acceptable values. For pH, a range of 6.0–7.5 is generally considered to be neutral to most aquatic organisms (Dodd 2010). The values for pH obtained in this study ranged from 7.10–7.65 which can be considered within the limits established as non-harmful. Also, there was no significant diferences between sites or control.

Total hardness, measured by concentration of calcium ions, ranged from 24-115 mg/l in all six water samples, which can all be classified as moderate (20-80 mg/l) except for Olho do Dueça which is classified as hard water (80-120 mg/l) (Dodd 2010). Calcium cations help to buffer the effects of acid pH and ameliorate the toxic effects of many dissolved metals, therefore moderate to hard waters tend to be adequate and even advantageous to amphibians (Ultsch et al. 1999).

Conductivity ranged between 154 and 499 µS/cm which are values deemed adequate for aquatic organisms in general (150-500 µS/cm) (Dodd 2010). This parameter reflects the water's ability to conduct an electrical current due to the total concentration of dissolved solids (TDS) present in the water which ranged from 70-351. For amphibian life, suggested levels of TDS range from 50 to 250 mg/l (Shaikh 2017) so Nascente de Ourão, Nascente de Anços and Olho do Dueça had higher values than recommended.

Concerning salinity on amphibian tadpoles, it is generally perceived as the lower the better (Dodd 2010). Salinity ranged from 0.03 to 0.23 PSU. Ponds or springs located near roads may also have an increase in salinity due to road salts, which pose a threat to amphibians (Karraker et al. 2008). Among other Iberian species, *Hyla meridionalis* for example tolerates low levels of salinity (García-París 2004).

Dissolved oxygen concentrations from all samples ranged from 8.45 to 9.25 mg/l which are well above the limit of 60-100% saturation deemed as stressful and even fatal for amphibian larvae under laboratory conditions (ASTM 2002) and is also above the limit imposed for minimum surface water quality for human consumption in Portugal (5 mg/l) (MA 1998).

When considering nitrogenous compounds, various studies have shown that these cause negative effects on amphibians, similar or greater than pesticides (Boone & James 2005; Egea-Serrano et al 2012). High levels of nitrates are known to inhibit growth, induce damage to the immune system and stress in most aquatic species (Romano & Zeng 2007). For instance, ammonium nitrate has a negative effect on larval size after exposure to concentrations of 45 mg NH_4NO_3/L (García-Muñoz et al 2011). Regarding nitrite effects on amphibians, a study with *Rana sylvatica* showed that exposure to concentrations of 0.5 mg/L NO_2^-N led to significant motality on early larval stages (Griffis-Kyle 2005). Nitrogenous fertilizers have been appointed as a contributing factor for the decline of *H. arborea* (Órtiz 2004). Levels of these fertilizers that are harmless in laboratory are likely to be more hazardous in the field because of potential synergetic interactions with other pollutants (de Solla et al 2002). However, quantification of nitrites and ammonium of sampled waters in this study were much lower (higher values of 0.04 and 0.29 mg/l respectively).

None of the above parameters are sufficient to give a plausible explanation for the higher mortality and reduced body lenght in site 4.

Fungicides, insecticides and herbicides cause a wide variety of negative direct ot indirect effects on amphibians and range from lethal, sublethal and chronic effects (Dodd 2010). One of the most commonly used broad-spectrum herbicide in the world is glyphosate (manufactured by Monsanto Company, USA). Of all the pesticides that were quantified in this study, only glyphosate presented concentrations above the detection limit in two of the sampled sites: Dolina dos Casais São Jorge and Dolina de Aljazede. Surprisingly, the site Dolina dos Casais São Jorge had a higher concentration of glyphosate (4.1 µg/l) compared to Dolina de Aljazede (0.028 µg/l) which presented a higher rate of mortality and reduced body size of the tadpoles. Water from Dolina dos Casais de São Jorge presented however the higher pH of 7.65, being slightly above the reference value for amphibian life (6.0-7.5) (Dodd 2010). Nonetheless, according to Relyea & Jones (2009), when exposed to a glyphosate-based herbicide (Roundup Original Max) two Hylidae species (*Hyla versicolor*, and *Pseudacris crucifer*) at larval stages had values of LC10 of 1400 and 100 µg/L and and LC50 of 1700 and 800 µg/L. These values are much superior to those found on the sampled waters in this study, even when compared to the values of LC10, given that site Dolina de Aljazede had 9% of mortality at a much lower concentrarion.

When regarding the insecticides analised (deltamethrin and chlorpyrifos-ethyl), it is to note that both were below the limit of detection and below both examples of LC50 in fish and amphibians, Canadian water quality guidelines for the protection of fresh water aquatic life established concentrations that are inferior to this limit (0.0004 and 0.0035 µg/l respectively).

Limited studies are available for comparing our results.

Biomarkers are useful tools to detect sublethal effects of exposure to contaminants, such as pesticides (Wu et al 2005) even when concentrations from samples collected from the field are below detectable limits (Fulton & Key 2001), as it happened in this case.

From the results obtained in this study, we verified that tadpoles exposed to water from Dolina de Aljazede - which had the higher mortality and reduced body lengths – had the highest level of GRed activity compared to all other sites.

The increase in GST activity may indicate a defensive reaction against the toxic and stress effects of pesticides (Güngördü et al 2016), since this enzyme has an important role in protecting the cell against xenobiotic substances through conjugation of many reactive xenobiotic's electrophilic substrates (Ferrari et al 2007). However, its inhibition is deemed as a failure of the detoxification processes (Cattaneo et al 2012). Nonetheless, a low activity may represent only low baseline activity combined with the absence of stressors. Studies on GST activity after exposure to some glyphosate based herbicides in amphibians have shown a decrease of this enzyme compared to untreated individuals (e.g. Lajmanovich et al 2011; Güngördü et al 2016), emphasizing the harmful effects of this compound. This may justify the slightly lower activity obtained for animals exposed to site 3 water, since glyphosate presented the highest values in this site.

Considering that the concentration of TBARS increases as a result of lipid peroxidation, indicating membrane damage, and even though there are no significant differences between the sites and the control, our results suggest that conditions in site 3,4,5 and 6 may not be completely appropriate for *H. arborea*. Such is related to the fact that lipid peroxidation for these sites was almost double when compared to the control animals.

3.5. Concluding remarks

Overall, water in Sicó massif regarding the presence and quantification of a few commonly used pesticides was below the detection limits, with the exception for glyphosate, and below the reference values for protection of aquatic life. This suggests that these water masses might be suitable for amphibians dweling in the karst massif, mainly in the early life stages. The exception might be for site 4 (Dolina de Aljazede), since it had influence on the growth of tadpoles which presented the smallest body sizes.

Nonetheless, and despite no patterns between lethal and sublethal effects were identified in our study, it is known that tadpoles raised in laboratory conditions are protected from many stressors that occur in the field and that are known to significantly augment the effects of contaminants, namely pesticides (ultraviolet radiation, predation, reduced food availability, inter- and intraspecific competition) (Davidson et al 2001; Releya & Mills 2001; Boone & Semlitsch 2002). Also, concentrations deemed sublethal or non-harmful in laboratory essays can potentially be more hazardous in field conditions (Mazanti 2003). Furthermore, the concentrations of glyphosate detected in the water migh not be the result of a recent application, which, being confirmed, can indicate that glyphosate concentrations can be much higher after recent applications, posing danger to these animals.

In order to ensure a proper habitat for breeding and larval development, which is fundamental for the conservation of local populations (Pellet 2005), as it is the case for local aquatic breeding amphibian species in Sicó massif, it is essencial to complement this study. Carrying our ecotoxicological studies in the field is likely to provide different results than those obtained in experimental laboratory analysis (Birge et al. 2000) and thus complement the already existing data.

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Chapter IV

4. Concluding remarks

This final chapter aims at providing an overall discussion of the previous chapters (II and III), integrating the results of both studies carried out in Sicó karst massif. The central aim of this work was to provide insight of amphibian ecology in karst environments, namely caves. Based on the results from chapter II, we verified an overall low abundance of amphibians in caves throughout the course of the surveys. However, it is important to underline that these results are demonstrative of observations made in a small window of time (November 2016 to February 2017) and surveys for each cave were only made once. Presence of amphibians in horizontal caves suggests an active search for a refuge while their presence in low-depth vertical caves is inconclusive. Despite this, other studies report the presence of amphibians in subterranean habitats for thermal refuges (Resetarits 1986) and breeding sites (Manenti et al 2016), namely *Bufo bufo* and *Triturus marmoratus* (Giménez-Lopéz & Guarner Deu 1982; Bonini et al 1999).

Considering that water availability is essential for amphibians, and that in the karst there is a very limited number sites with available surface water, it is important to assess the suitability of those sites for amphibians, namely in the most vulnerable early life stages. The work in chapter III was carried out with this in mind, and sinkhole ponds and springs from the Sicó massif were assessed for their suitability to host early life stages of amphibians. From the sites assessed only the water from Dolina de Aljazede had a negative influence in the growth of tadpoles. Furthermore, for the abiotic parameters determined and for the pesticides analysed, the majority of the sites presented values within the range of the benchmark values for water quality. The exception occurred for the quantified pesticides and for Dolinas dos casais de São Jorge (site 3) which presented a higher concentration of glyphosate.

Based alone on the quantification of pesticides, the results would suggest that, from the six sites sampled, the site 3 would be the most inappropriate for the early life stages of amphibians. Nonetheless, the biological approach allowed to perceive that the water from site 4 is the one that impairs the most the early life stages these animals. Also, the results for lipid peroxidation show that, when compared to the control, the values obtained for site 3, 4, 5 and 6, are twice as high. Even though no statistical differences where found, this could suggest that the suitability of the water from these sites is not the best for the development of the early life stages of amphibians. Considering that, in karst areas, sites with surface water can be scarce and that surface water is essential for the survival and reproduction of the majority of local amphibians it is pivotal to assure their suitability for these animals.

Therefore, ensuring a proper habitat for breeding and larval development remains fundamental for the conservation of local populations (Pellet 2005), as it is the case for local aquatic breeding amphibian species in Sicó massif. The European tree frog, as with many other amphibians that breed in ponds, are exposed to both aquatic and terrestrial habitats during different stages of their life cycle. Hence, it is necessary to conduct further investigation that focuses on potential contamination of both habitats and on the potential toxic effects of pesticides and metals on local aquatic breeding amphibian species in Sicó Massif but also in other Portuguese karst areas.

Moreover, ecotoxicologic investigations in the field are likely to provide different results than those obtained in experimental laboratory analysis (Birge et al. 2000), so further studies on field conditions are sure to provide more insight on this subject.

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